Investigation into the synthesis of biodegradable polymers for use as drug targeting agents

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INVESTIGATION INTO THE SYNTHESIS OF BIODEGRADABLE POLYMERS FOR USE AS DRUG TARGETING AGENTS.

Thesis
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The research contained within this thesis has been carried out in the School of Pharmacy and Pharmacology under the supervision of Dr. Michael D Threadgill and Dr. Colin W Pouton.

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INVESTIGATION INTO THE SYNTHESIS OF BIODEGRADABLE POLYMERS FOR USE AS DRUG TARGETING AGENTS.

ABSTRACT.

Macromolecular drug carriers offer the potential to deliver inherently toxic drugs in a site-specific, controlled manner. However, once the function of the carrier is over it should be expelled from the cell. Soluble polymers are captured by pinocytic uptake, rendering them lysosomotropic. It is proposed that co-polymerisation of PEG with alternating oligopeptide sequences would afford a biodegradable polymer. The peptide sequences are selected for sensitivity to hydrolysis by lysosomal enzymes. Poly(ethyleneglycol) (PEG) is biocompatable and may infer beneficial properties onto the PEG / peptide conjugate such as , a reduction in immunogenicity, and increase in solubility and plasma half-life. The soluble macromolecule would be stable in body fluids but could undergo complete degradation into small chains within the lysosomes of the target cell.

The oligopeptide sequences GlyGly, PheLeuGly and GlyPheLeuGly were synthesised by solution methods between (benzyloxy)aryl structures. For example, 2-(4-benzyloxyphenyl)ethylamine was coupled to BOCglycine using DCC. Treatment with HCl afforded the de-protected amine salt. Coupling of this amine with BOC protected amino acid and dipeptide active esters, served to extend the peptide chain in an iterative process. Final deprotection (HCl) was followed by coupling with N-(4-BnOC_6H_4CO)GlyOPFP to give the α,ω-bis(OBn) peptides. Hydrogenation effected debenzylation. The phenolic hydroxy groups were subsequently converted to the glycidyl ethers by treating with epichlorohydrin. The copolymerisation of α,ω-bis(methylamino) PEGs with α,ω-bis(glycidyl) peptides was chosen as the polymerisation strategy towards the alternating PEG-peptide linear polymers. To provide the appropriately derivatised monomer the PEGs were converted to the α,ω-bis(chloroformate) with phosgene. Treatment with BOCSarNH$CH_2CH_2NH_2$ gave the corresponding bis(urethane). Deprotection with HCl gave the required α,ω-bis(methylamino)-derivatised PEG. By introducing a secondary amine onto the PEG the risk of polymeric cross linking was reduced. The PEG containing polymers were functionalised further by the inclusion of glutamic acid within the oligopeptide sequence. The glutamate side chain may be used to attach cytotoxic drugs or targeting moieties in the future.

To My Parents ...

...and they stole into the city within a wooden horse.....

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List of abbreviations

ACTH	adrenocortico thyroid stimulating hormone
ADR	adriamycin
AF	asialofeutin
Ala	alanine
ara-A	arabinofuranoside
BOC	1,1-dimethylethoxycarbonyl
BSA	bovine serum albumin
CALLA	common acute lymphoblastic leukaemia antigen
CEA	carcinoembrotonic antigen
DIVEMA	divinylether maleic anhydride
DKP	di-ketopiperazines
DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DNM	daunomycin
DNR	daunorubicin
EGF	epidermal growth factor
EGF	extracellular growth factor
EPR	enhanced permeability and retention effect
Gly	glycine
hCG	human chorionic gonadotrophin
HDL	high density lipoprotein
HOBt	1-hydroxybenzotriazole
HPMA	N-(2-hydroxy-propyl)methacrylamide
IL-2	interleukin-2
KDa	kilodaltons
LDL	low density lipoprotein
Leu	leucine
LHRH	luteinising hormone releasing hormone
Lys	lysine
M-PEG	monomethoxypolyethylene glycol
mAb	monoclonal antibody
mAb-PS	monoclonal antibody-photosensitiser conjugate
MCPBA	*meta*chloroperoxy-benzoic acid
MMC	mitomycin C
MMC-D	mitomycin C-dextran
$MMC\text{-}D_{an}$	mitomycin-D *anionic* conjugate
$MMC\text{-}D_{cat}$	mitomycin-D *cationic* conjugate
MSH	melanocyte stimulating hormone
MTX	methotrexate
MVE-2	modified DIVEMA
NCS	neocarzinostatin
P	amino acid residue

p(HEMA)	poly(2-hydroxyethyl)methacrylate
PBLG	poly(γ-benzyl)-glutamate
PDT	photodynamic therapy
PEG	poly(ethyleneglycol)
PEG-rG-CSF	poly(ethyleneglycol) recombinant granulocyte colony stimulating hormone
PEG-SOD	poly(ethyleneglycol) - superoxide dismutase
PEO	poly(ethyleneoxide)
PEO-NCO	poly(ethyleneoxide) - isocyanate
Phe	phenylalanine
PLL	poly-L-lysine
PQ	primaquine
PVA	poly-vinyl-alcohol
PVP	poly(vinylpyrrolidone)
RES	recombinant human interferon
rIL-2	recombinant interleukin 2
RNA	ribonucleic acid
RSA	rabbit serum albumin
S'	enzyme subsite
SA-DNR	serum albumin - daunorubicin
SMA	styrenemaleic acid
SMANCS	styrene-co-maleic acid neocarzinostatin
THF	tetrahydrofuran
Ts-mAb	tumour specific antibody
TSA	tumour specific antigen

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Chapter 1. Introduction.

1.1. Introduction.

1.2. Drug-Polymer Conjugates:- potential for improved chemotherapy?

The work presented in this thesis concerns the design and synthesis of novel, biodegradable, polymer carriers for drugs or diagnostic reagents. It is an attempt, primarily to improve on the existing methods of drug delivery and targeting. The introduction begins by asking if any improvement is required in present day chemotherapy. Further sections discuss key concepts including the use of controlled drug delivery to achieve better biodistribution and activity. The general principles of a drug carrier are reviewed with pertinent examples of polymer and drug targeting moieties. A final comprehensive section critically assesses the use of polyethylene glycol as a therapeutic agent and discusses it's use as a soluble, polymeric drug carrier.

Over the past 40 years cancer chemotherapy has evolved significantly. Opinions on the overall success of chemotherapy are often conflicting. They range from the pessimistic view that because cancer mortality has remained essentially unchanged since 1954 (1), chemotherapy has been an unqualified failure, to the optimistic view that most cancers can now be treated in some way offering the chance of remission and an overall improvement in the quality of life. The reality lies somewhere between the two. In all malignant conditions therapy may involve surgery, radiation, immunotherapy, chemotherapy or a synergistic combination of treatments. Of the above, chemotherapy has played an important and effective role in treating both solid and haematological malignancies.

Despite efforts to develop novel cytotoxic drugs and treatments, progress is being made in small steps with no effective chemotherapy available to date for certain solid tumour types and advanced tumours. In addition, the therapeutic indices of most anticancer drugs in systemic chemotherapy still remains marginal with no site specificity, and inhibition of tumour growth is invariably accompanied by serious damage to proliferating normal host cells. Of particularly relevance to this is the destructive effect on the rapidly dividing cells of the haematopoietic system, gastrointestinal tract as well as the hair follicles which can lead to alopecia and a reduction in patient self-esteem.

Growing interest in the drug-polymer field has in part been due to this limited progress in the successful development of effective new drug entities, but also due to the realisation that new approaches must be adopted if we are to see an improvement in therapeutic activity. The efficiency of drugs would increase enormously if we were able to direct them selectively to their cellular targets. This dream was echoed by Paul Ehrlich (2) who in 1906 predicted the application of antibodies as "bodies which possess a particular affinity for a certain organ .... as carriers by which to bring therapeutically active groups to the organ in question". The concept of Ehrlich's "magic bullet" is deceptively simple:- get the right amount of the active agent at the right time to the right place. These ideas were not tested experimentally until the mid 1950's when Mathé (3) attempted to target methotrexate (MTX) to L1210 leukaemia cells in the mouse. It is, however, work on the use of polylactic acid by Yolles *et al* (4) that is regarded as initiating the avalanche of practical advancements seen over the last twenty years. Since then many different approaches to drug delivery have been explored, within a diversity of scientific fields. This reflects the inter-disciplinary nature of macromolecular research.

Although polymer chemistry now occupies a central role, with the use of polymer-based technologies for improved delivery of cancer chemotherapy, it has not been as widely

explored as the liposomal and antibody-based strategies. This is disappointing as polymers have been extensively studied and are already in daily use in many biomedical applications including, contact lenses, plasma expanders, wound dressings and pharmaceutical excipients. In addition, research into new polymers for implantable, or insertable controlled release devices, targeted drug carriers and transdermal systems shows great pharmaceutical potential. To this end powerful commercial interests have tended to fuel this area of research and this is a situation which is reflected by the number of collaborative programmes between scientists in academia and industry.

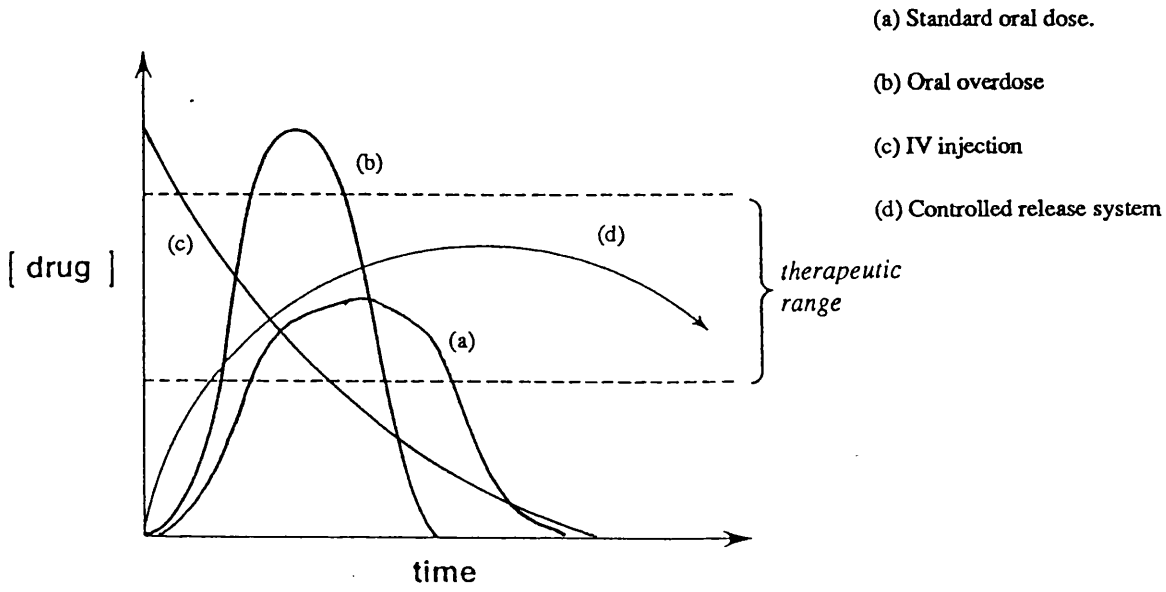
There have already been a number of commercial successes. Zoladex ®, the polylactide-co-glycolide subcutaneous implant delivering a luteinising hormone releasing hormone (LHRH) over a 28 day period is used for treating prostate cancer (5), and a poly anhydride matrix containing carmustine is currently in phase III evaluation for treatment of glioma multiforme (6). Soluble polymer conjugates comprising of monomethoxy polyethyleneglycol (M-PEG) have been bound to various proteins including asparaginase (7) and interleukin II (IL-2) (8) and await approval for their use in cancer chemotherapy. Initial results with another polymer-protein conjugate, styrene maleic acid (SMA)-neocarzinostatin (NCS) or SMANCS, evaluated in more than 500 patients presenting with primary hepatomas or secondary tumours of the liver (9) are very encouraging.

1.3. The Concept of Controlled Release v The Concept of Drug Targeting.

The objective in designing a controlled release system is to liberate a pharmacologically active agent in a predetermined, predictable and reproducible fashion. Such formulations are generally very useful and exhibit few side effects as the drug circulates systemically at an optimum level for a prolonged period of time (*Figure 1.1*). Bolus administration of an antitumour agent typically leads to high peak concentrations in the

plasma, tumour and unfortunately also in sites of toxicity. With the use of controlled release formulations the peaks and valleys of plasma concentration are eliminated. This extends the time the effective therapeutic dose is present at the target site which is important as many antitumour agents express differential activity throughout the cell cycle. The premise of targeted drug delivery is the assumption that the therapeutic index of a drug can be improved when the drug accumulates selectively in specific tissues, organs or cell types. Drugs can be targeted by a wide range of mechanisms. However, both the targeted and the controlled concepts of drug release share the potential to decrease the dosage of drug required by improving the pharmacokinetics and localisation of the drug respectively. This is important when expensive recombinant enzymes or active ingredients are involved.

Figure 1.1. Theoretical plasma concentration after administration of various dosage forms.



There are two fundamental approaches to the targeting of drugs. On a very basic level one can differentiate between insoluble, particulate drug carriers and the soluble macromolecular drug conjugates. Furthermore one has to distinguish between actively and passively targeted systems. Briefly, passive targeting takes advantage of an existing body

process, *e.g.*, the rapid accumulation of particles < 1 micron in the liver or spleen after i.v. injection. In contrast, during active targeting the natural tendency of the body to distribute the active substance is altered due to targeting moieties on the drug or carrier.

Polymeric controlled release systems can be placed into virtually any of the available body cavities. In addition they can be implanted or attached externally to the skin, allowing a considerable choice as to the route of drug administration. Crude targeting can be achieved by introducing a polymeric matrix or reservoir system adjacent to a tumour, where the tumour can be exposed to a localised, continual drug exposure. Both passive and active targeting have been used to deliver chemotherapeutic agents. This will be reviewed in Section 1.11.

1.4. Characteristics of the Drug Carrier System.

Trouet (10) defined a carrier system as "a molecule or an assembly of molecules, the major characteristic of which is that it is unable to cross (native or bound to a drug) the plasma membrane of eucaryotic cells by permeation or active transport". The carrier requirements of different therapeutic agents will vary widely, but most drugs are restricted by their lack of selectivity towards their respective targets. Macromolecular drug carrier systems are being developed in an attempt to alter the tissue localisation of drugs (11). This would enhance the effects of a drug at a desired site of action relative to the unwanted side effects. The application of such carriers to chemotherapy where extremely toxic agents are used, is clearly an advantage although other disease states, *e.g.* viral, protozoal infections, emphysema and arthritis could also benefit from selective drug delivery. The *in vivo* distribution of a carrier linked drug will largely depend upon the properties of the carrier and will therefore differ from the distribution of the free drug given by the same route (11).

There are certain criteria that must be considered for a macromolecular carrier:-

(a) the carrier should be easily prepared in a homogenous, cost effective manner;

(b) the carrier should be biocompatible and it's metabolic products should lack any intrinsic toxicity;

(c) the chemical reactivity of the carrier should be suitable for attachment of the drug, and should have a high drug carrying ability;

(d) the carrier should be biodegradable, and not show substantial accumulation in the body;

(e) the carrier molecule may be a natural substrate for a physiological receptor;

(f) the carrier may have influential physical properties. One needs to consider whether it is a compact or extended molecule?. Is the molecular weight smaller or larger than the renal threshold?, What are it's solubility and electrical charge?.

The following is a list of objectives which may be achieved using a drug or enzyme carrier system:-

(a) stabilisation of a drug or enzyme in it's active form, thereby retarding normal biodegradation;

(b) improved localisation *via* passive or active targeting;

(c) release of the active drug, either chemically or enzymatically, at a controlled and predictable rate;

(d) increased circulation half-time;

(e) minimisation of non-specific cytotoxicity;

(f) enhancement of solubility.

1.5. Drug Carriers.

A comprehensive review of the literature relating to controlled delivery and targeting, revealed several hundred publications. Many different systems have been explored including soluble low molecular weight prodrugs (12), polymeric implants (13), and not least the use of devices such as infusion pumps (14). Macromolecular and cellular (or particulate) drug carriers have been extensively investigated, with macromolecules forming the basis of the majority of systems. Examples of insoluble particulate carriers include microspheres (15), nanoparticles (16), liposomes (17, 18, 19), emulsions, and cellular drug carriers, for example, the erythrocytes (20) and leucocytes (21). Such delivery systems may incorporate magnetic control to localise the drug carrier complex in a certain area of the body (22). However, the micro-particulate carriers will not be discussed here as they are neither soluble, degradable or readily targeted. Instead, the reader is directed to the excellent reviews cited.

Considering the effort expended in the area of macromolecular conjugates, the number of reports of improved efficacy has been quite low. This section will review the benefits and shortcomings of some of the key macromolecular drug delivery systems available today. Detailed accounts concerning the methods used for the coupling of drugs to their carriers will be omitted as it is beyond the scope of this thesis. An exhaustive review on the coupling procedures of PEG is included in *Section 1.13*.

1.6. Macromolecular Drug Delivery Conjugates.

Macromolecular carriers can be divided into a number of categories. The first class, the natural polymers, include protein carriers (antibodies, albumin, glycoprotein, lectin, hormones), dextran and deoxyribonucleic acid (DNA). The second class is comprised of the

synthetic polymers such as N-(2-hydroxy-propyl)methacrylamide (HPMA) and may include some pseudo-synthetic polymers such as the polyamino acids, *e.g.*, poly (lysine).

1.7. The Natural Carriers.

Natural carriers, by their nature are derived from sources which have existed long enough for mechanisms to evolve that utilise them as substrates. For this reason many of them are susceptible to degradation, thus facilitating metabolic removal from the organism after administration. This is an advantage as it prevents the accumulation of the carrier material within the cells once the drug has been delivered. This process is known as thesaurosis, and can lead to an immune reaction and toxicological problems. Natural polymers were pioneer degradable drug carriers but one disadvantage was that they were frequently recognised as foreign *in vivo* and induced immunogenic problems (23). In addition, as products of biosynthesis their structure and properties could hardly be shaped according to clinical needs.

1.7.1. Proteins.

1.7.1.1. Monoclonal Antibodies.

The coupling of a drug to a monoclonal antibody (mAb) that can bind to specific cell surface antigens has great promise for directing cytotoxic agents to appropriate sites. In 1958, Mathé and co-workers (3) were successful in treating a mouse leukaemia (L1210) with diazo linked conjugates of the antitumour drug methotrexate and hamster antibodies prepared against the L1210 cells. Despite this, thirty five years later, the use of antibodies in routine clinical practice still remains as elusive as Ehrlich's magic bullet. The problems are many fold. From Mathé's results it is not possible to determine whether the antibody was

actually delivering the drug to the tumour site or whether it was simple altering either the pharmacokinetics of the drug delivery or enhancing the toxicity of the drug molecule by altering the properties of the tumour cell.

The possibility of antibody drug synergism was re-investigated in the 70's by conjugating the alkylating agent, chlorambucil with an anti-tumour antibody. It was reported (24) that the growth of transplanted tumours was prevented in conditions under which equivalent amounts of either free drug or free antibody were ineffective. However, further study (25) showed that protection against tumour growth was as effective as when the drug and antibody were injected separately. Rubens and Dulbecco (26) postulated that the antibody was not therefore directing the drug to the target but that each acted synergistically to increase the susceptibility of the tumour. This synergism has also been demonstrated with the anti-tumour agent cytosine arabinoside (27).

The discovery of tumour specific antigens (TSA) (28) allowed the large scale production of selective tumour antibodies. Gilliland *et al* (29) coupled the diphtheria toxin *via* a disulphide linkage to a mAb directed against a colorectal carcinoma tumour associated antigen. The conjugate showed nearly 100% cytotoxicity to the colorectal cells *in vitro* and did not affect the cells which lacked the antigen. The danger with the use of a mAb preparations against a single antigenic determinant is that some cells within primary or metastatic cancers may not possess, or may have lost that specific antigen, thereby escaping the antibody-drug conjugate (30). This typical pattern is termed "patchwork" and may be due to the cell cycle-dependent expression of some cell surface tumour antigens. In addition, circulating tumour specific antigen may bind antibody or antibody-drug complexes in the serum before they have a chance to attack the target tumour cells. Similarly, Mach *et al* (31) found the use of antibodies against carcino-embryonic antigen (CEA) difficult to use in diagnostic situations due to the presence of high levels of circulating CEA in the serum.

The use of highly selective mAb-directed treatment may be further compromised by cross-reactivity due to shared antigens on normal tissues or by non-specific tissue uptake (32). Heterogeneity is a characteristic shared by all human carcinoma-associated antigens. This is manifested in a variety of ways. Often the tumour antigens may be integral components of the cell membrane and do not undergo internalisation after antibody binding. In addition, there may be a significant quantitative difference with respect to the antigen density per cell.

Recently the above problems were addressed by utilising mAb's that carry light-activated molecules (photosensitizers, *e.g.*, chlorin e_6), (*see Section 1.12*). These conjugates are innocuous without illumination but produce reactive species, such as a singlet oxygen ($^1O_2$), upon absorbing light. By confining illumination to areas containing specifically bound photosensitiser, toxicity can be limited to target cells only (33, 34). This dual selectivity (mAb binding and illumination) means the mAb need not be completely tumour selective. As long as the cross-reactive or non-specifically bound mAb-photosensitiser (mAb-PS) is not illuminated there will be no adverse effects to normal tissue. In contrast to most conventional cytotoxic drugs and toxins the mAb-PS need not be internalised to have their effect. Diffusion of the potent singlet oxygen can produce a cytotoxic effect in cells to which it is only loosely associated. In addition neighbouring antigen-negative tumour cells which are not bound to the mAb-PS conjugate may also be killed. Oseroff *et al* (35) found that haematoporphyrin molecules could be directly coupled to the carbohydrate moieties on the Fc portion of mAbs with no effect on the antigen binding site and permitting the mAb to exert highly selective phototoxicity. However, with direct attachment, less than 5 chromophores could be linked to the mAb. To increase this ratio, with a concomitant increase in toxicity, dextran-dye conjugates were prepared and linked to the mAb carbohydrate. The authors found that unconjugated chlorin e_6 under the same conditions was not phototoxic (33, 36).

Vitetta and colleagues (37) have questioned the accessibility of solid tumours to immunotoxin therapy due to their dense connective tissue component. One of the problems associated with the use of mAb is their relative large molecular weight (IgG = 150 KDa). Conjugating a drug to the mAb will increase the molecular weight still further. This presents an important obstacle to diffusion of the conjugate through the vasculature and subsequent loss of material to the reticulo-endothelial system (RES). The vasculature does not normally have receptors capable of recognising this large conjugate (38) and despite the inherent leakiness of tumour vasculature there is often a poor localisation of the mAb-conjugate. This is reflected in human tumours where only 0.005% of the mAb administered can be found at the target (39) and much of this is shown to be confined to the periphery of the tumour mass (40, 41). This led Poznansky and Juliano (42) to propose that bound vasodilators such as histamine could be administered to facilitate the penetration of the blood tissue barrier. Another approach is through the administration of a biological response modifier, such as recombinant human interferon (rH-IFN) , which can result in amplifying the cell surface tumour antigen expression. By increasing the level of mAb binding to the tumour cell population more effective therapy may result.

There are reports in the literature of antibodies that have been bound to a number of highly toxic substances. With a concomitant rise in toxicity the amount of effective mAb-conjugate at the target site need not be so high and problems of localisation are reduced. Unfortunately, experiments with the toxin, ricin (A chain), have shown that the therapeutic index of the immunotoxin must be improved if any significant tumour burden is to be challenged. Masuho *et al* (43) demonstrated *in vitro* that the mAb-CALLA (common acute lymphoblastic leukaemia antigen) conjugate appeared much less toxic than free ricin. Other workers report similar findings, (44) and suggest this may be due to the inability of certain antibodies to induce endocytosis either because of low binding affinities, or because the target antigen may be one which is either shed or not readily internalised. The reduction in

binding affinity may be due to an alteration in the tertiary structure of the antibody during drug binding (45). This loss of binding activity and consequently toxicity can be reduced if the antigen-binding site is blocked reversibly before drug conjugation takes place (46). The difficulty in coupling adequate amounts of drug to an antibody without denaturation is a problem. The use of low molecular weight toxins as opposed to large conventional cytotoxic drugs solves this problem to a certain degree. However, this may not be totally acceptable since uptake of even tiny amounts of the toxin by healthy cells, through non-specific pinocytosis, could be lethal.

1.7.1.2. Albumin.

The biocompatibility of albumin is established. Albumin lacks toxicity and any intrinsic site selectivity (47) and therefore specificity must be provided in some other way. Chu and Howell (48) used this lack of site selectivity to their advantage when they bound MTX to bovine serum albumin (BSA) and injected this into the peritoneal cavities of BDF mice with the L1210 tumour. The attachment of the drug to the high molecular weight carrier led to the prolonged localisation of MTX in the ascitic fluid together with elevated intracellular MTX levels after 24 hours. By decreasing the clearance of the drug from the compartment, the total drug exposure in the tumour was increased and exposure to normal cells was decreased. In contrast, it was reported (49) that MTX-BSA was 50 - 100 fold less toxic to L1210 cells in culture than was free MTX. The reasons behind this discrepancy lie in the large molecular size of the conjugate. Rapidly proliferating cells, such as those found in tumours, often have increased pinocytic uptake rates. The *in vitro* study essentially compared the uptake of free MTX to that of the bound MTX. The former is normally taken up *via* facilitated transport mechanisms (48), whereas the latter, being much larger, is incorporated into the cell by fluid phase pinocytosis, which is a much slower uptake mechanism. Hence the free MTX appeared to have a more rapid, toxic effect compared to

the conjugate. During *in vivo* experiments, the bulky conjugate is confined by physiological barriers, *i.e.* membranes, to the compartment into which it is injected, where it has a localised effect as described above. Free MTX, on the other hand, is able to penetrate the vasculature and is removed from the site of injection rapidly. In addition the short half-life of the MTX *in vivo* reduces the apparent toxicity of the drug. Intraperitoneal injections of BSA-MTX were also found to be superior to free MTX against Lewis lung carcinoma and subcutaneous implanted solid tumour in rats and mice, where an increased life span was observed, and the number and size of metastatic nodules was reduced (50).

Albumin has been used as a drug carrier in other instances, *e.g.* Amantin, 5 fluoro-deoxyuridine and cytosine arabinoside have been coupled to rabbit serum albumin (RSA) (51, 52). More topically, daunorubicin (DNR) was linked to succinylated albumin using peptide spacers (53). Following incubation of the drug conjugate with lysosomal enzymes *in vitro* it was shown that the tetrapeptide spacer (Ala-Leu-Ala-Leu) was cleaved most readily, with 74% of DNR being released after 10 hours. The possible use of these conjugates for the delivery of cytotoxic drugs to the lysosome encouraged the authors to test the chemotherapeutic activity of the various SA-DNR conjugates on L1210 leukaemia *in vivo*. Both *in vitro* and *in vivo* experiments revealed that the conjugates showed less toxicity than the free DNR.

Lactosaminated serum albumin (L-SA) is reported (52) to bind to galactosyl terminated glycoprotein specific receptors, on hepatocytes. This conjugate was devoid of immunogenicity, even when coupled to arabinofuranoside (a simple sugar) and shows potential as a hepatocyte-specific drug delivery system.

1.7.1.3. Other Proteins. Glycoproteins, Lectins and Hormones.

The use of glycoproteins (54), lipoproteins (55), lectins (56) and hormones, particularly melanotropin-drug conjugates (57) as drug carriers has not been extensive. This is in general due to the highly selective and ubiquitous nature of their respective receptors, making organ or tissue specificity difficult. In the case of melanocyte stimulating hormone (MSH) which could be used to target melanoma, this has been considered tolerable as the only side effect should be non-fatal de-pigmentation. Daunomycin (DNM) was linked to MSH and found to be three times more toxic to melanoma cells *in vitro* than free DNM (57).

However, *in vivo* experiments were not successful and the conjugate showed toxicity to the liver and kidneys. These findings reflect the possibility that melanocytes were not the only non-target cells possessing MSH receptors. Also a possible problem in all receptor-mediated site-specific drug delivery systems is that endogenous ligands can compete for a finite number of receptors on the target cell. Anderson *et al* (58) demonstrated the uptake of cholesterol-labelled or protein-labelled HDL by rat adrenal cells, only after administration of ACTH had caused depletion of the circulatory pool of cholesterol.

Exposure of the terminal sugar residues on glycoproteins reveals asialoglycoproteins (59) which are recognised and rapidly cleared from the circulation by hepatocytes (galactosyl groups) and macrophages (mannosylated ligands) (60). Hepatocytes are targets for various infective agents particularly those producing hepatitis and malaria. To this end galactosyl-terminating glycoproteins have been used as hepatocyte-specific drug carriers, namely asialofeutin (AF) (59). Coupling of trifluorothymidine (F_3T) and adenine-9-arabinofuranoside (ara-A) to AF did not interfere with the binding of AF to surface receptors on the hepatocytes and the results in mice injected with *Ectromelia* virus (infection

of the Kupfer cells) were optimistic. However, ara-A conjugates of AF have shown immunogenicity which could lead to inactivation of the conjugates, rapid clearance from the circulation and allergic reactions. Trouet *et al* (53), in some pioneering work, succeeded in coupling primaquine (PQ) (an anti-malarial agent) to AF *via* a tetrapeptide spacer, Ala-Leu-Ala-Leu-PQ. Selective hepatocyte endocytic uptake of the conjugate together with intracellular degradation within the lysosomal compartment resulted in an increased hepatocyte concentration of PQ over the free drug. The theoretical applications of this initial research will be discussed in later chapters.

It is generally accepted that neoplastic cells have an increased endocytic rate compared to normal cells. In conjunction with this there will be an increased turnover of cell surface receptors that are continually being internalised. In 1981 Gal *et al* (61) suggested this process could be used to achieve preferential site selectivity of LDL-drug conjugates enabling more drug to be internalised. Other workers (62) demonstrated the internalisation of fluorescent lipoprotein preparations in cultivated human fibroblasts *via* receptor-mediated endocytosis of LDL and recently this receptor has been used extensively by Geze *et al* (63) to deliver porphyrin loaded LDLs to the lysosomal compartment in order to investigate photochemical reactions within the lysosomes. In contrast, the compound benzo(a)pyrene incorporated into LDL was shown to enter these cells by a mechanism independent of the LDL receptor complex (64). This questions the usefulness of LDL as a selective drug carrier in certain situations. Another example of receptor-protein interaction was cited by Chang *et al* (65). The toxic sub-units of the diphtheria toxin were coupled to a human placental lactogen and epidermal growth factor (66). The conjugate was able to bind effectively to lactogenic receptors. However, upon binding, the receptor was unable to mediate entry of the toxin into the cells. This point is an important concept in drug targeting, since often the drug must be internalised to exert it's biological effect. More positive results were obtained with ricin conjugates. When linked to human chorionic

gonadotrophin (hCG) (67) the conjugate showed selectivity for rat Leydig cells possessing receptors for the β subunit of hCG. However, cell kill was not as efficient as with free ricin.

The use of lectins as drug carriers has largely been ignored due to their lack of ligand selectivity and adverse interactions with body tissues (56). At doses as low as 10 μg intense inflammatory response with oedema and erythema occurs at the site of injection. Lectins are proteins that agglutinate cells by binding to specific carbohydrate residues. Due to the ubiquitous distribution of sugar residues *in vivo*, lectin usually remains at the injection site as a result of interactions between the sugar-binding receptors on the lectins and sugar residues present at the injection site. In terms of localised therapy this regimen may be advantageous, and some authors (68) have suggested using the non-toxic lectin from the tomato to act as a bioadhesive in anchoring drug-bearing microparticles at a certain site (69). Preliminary studies have shown that it's use improves the bio-availabilty of oral drug delivery by adhering to the gastrointestinal tract of the rat.

1.7.2. The Polysaccharides. Dextran.

The polysaccharides dextran, inulin, cellulose and starch are frequently used as drug carriers. Dextran is a collective name for a class of bacterial (*Leuconostoc mesenterrides*) polysaccharides composed of (1 - 6)-linked-α-D-glucopyranosyl residues [*see Figure 1.2(a)*]. Native dextran has a molecular weight reaching several hundred million and a high polydispersity. Commercial dextran fractions with a well defined range of molecular weight are prepared by controlled hydrolysis and repeated fractionation with fractions of average molecular weight 40, 70 and 110 KDa being used for blood plasma substitutes.
The reader is directed to the many reviews on the activation and biodegradation studies of dextran (70, 71). The advantages of dextran as a drug carrier are:- (1) high water solubility; (2) well characterised and repetitive chemical structure; (3) availability of different molecular weight fractions ranging from 2×10^3 to 10^6; (4) low toxicity and low pharmacological

activity; (5) protection of conjugated drugs or enzymes from biodegradation. The clinical use of dextran as a plasma expander makes it very popular as a drug carrier (72, 73) or as a polymeric spacer between drug and carrier macromolecules. In this latter use dextran is usually employed as the linking polymer between a drug and a tumour-specific antibody (74) [*see Figure 1.2b(b)*] to direct antitumour drugs or boron compounds (used in neutron capture therapy) (75). Drugs or enzymes can be coupled to the hydroxyl groups of dextran in a variety of ways, promoting the versatility of dextran as a drug carrier. Linking drugs or enzymes to dextran generally confers greater chemical and biological stability to the conjugated compound, as in the case of methotrexate (76), insulin (77), haemoglobin (78) and procainamide (79).

In 1978, the potency of daunomycin-dextran conjugates using periodate-activated dextrans was investigated (80). Experiments *in vitro* on YAC cells were disappointing as they showed that at low dosages the conjugate was less active than the free drug. On the contrary, evaluations *in vivo* of these conjugates in mice bearing YAC lymphoma cells revealed that at high doses (20-25 mg / kg), the conjugates could completely prevent tumour growth. In this dosage range, the free drug was very toxic, highlighting the advantage of the repeated use of a drug-carrier over the parent drug.

A general mistake made when developing drug carriers is the assumption that all the properties which were tested with free carrier polymer will be retained after the modifications necessary for drug binding, and after attachment of various ligands to the macromolecule. Firstly, the degradability of dextran depends on natural branching (81), with the bonds in the vicinity of branch linkages and at the ends of the chain being more resistant to enzymatic attack by dextranase. Any modification of these branches might further increase the stability of the bonds and may decrease the susceptibility to degradation (82). This was demonstrated neatly when Hurwitz *et al* (83) covalently coupled

daunorubicin *via* the keto group to dextran hydrazide yielding products with a potentiated antitumour activity in mice which had received 105 YAC cells. This group demonstrated that binding of the drug to dextran *via* a non-hydrolysable sulphide bond resulted in a loss of antitumour activity *in vitro* and *in vivo*. This suggests, therefore, that in order for dextran-daunorubicin derivatives to have retained antitumour activity, the drug-carrier linkage must be cleavable. This is in contrast to the work with aminoethylated dextran and methotrexate by Chu and Whiteley (84, 85). Here, it was found that the carrier bound drug retained the ability to interact with dihydrofolate reductase. The dextran derivative also persisted for longer time periods after intra-peritoneal administration in BDF_2 mice when compared to a similar group of mice treated with free methotrexate.

Fig 1.2(a). Dextran.

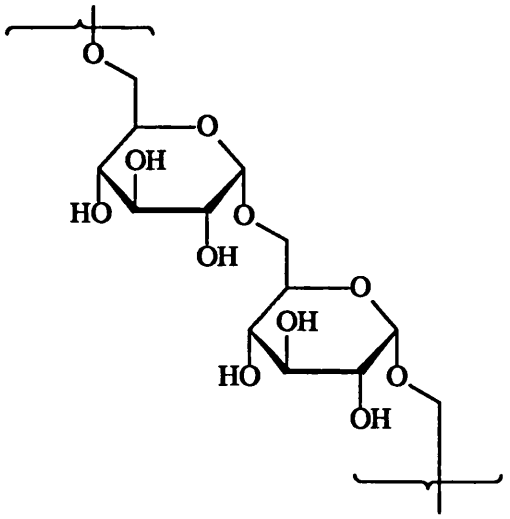
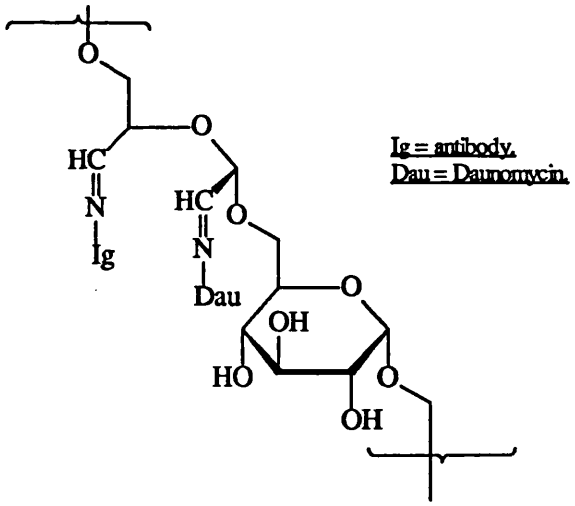


Fig 1.2(b) Drug is linked to an immunoglobulin via a dextran spacer group.



Ig = antibody.
Dau = Daunomycin.

Many authors consider that the immunological properties of the polysaccharides have not yet been fully clarified. Although immunological responses to dextran are rare, adverse reactions to repeated injections have been reported, including haematological

changes and inflammatory reactions (86). Further research has suggested that the immunogenicity of dextrans may be affected by a high degree of modification with different functional groups or residues. The introduction of a chain of six to eight residues into the dextran polymer substantially increased the immunogenicity of dextrans, and Rihova and Riha (88) included dextran in a review of the immunological problems associated with polymer-bound drugs. They indicated that the immunogenicity of polysaccharides is dependent upon the molecular size of the polymer, the resistance of the polymer to enzymatic cleavage and the injected dose.

1.7.2.1. Dextran and Charge.

Positively-charged macromolecules such as protamine and poly-L-lysine (PLL) have been reported to show high cellular interactions. The poly-cationic mitomycin-polylysine conjugate, for instance, is more strongly adsorbed to tumour cells than many conjugates which have a negative (anionic) charge (89). To this end, the cytotoxic agent, mitomycin C (MM-C) has been coupled to dextrans of various molecular weights (90), using hexanoic acid as a spacer. The physicochemical properties of the dextran conjugate, *e.g.* the net electrical charge and the rate of release of the drug from the conjugate, were controlled by changing the structure and / or the method of introducing the spacer linkage to the dextran backbone. A cationic conjugate, MMC-D_{cat} was found to rapidly accumulate in the liver, spleen and lymph nodes after intravenous administration of the drug (91), while the interaction between anionic MMC-D_{an} and hepatocytes was negligible. The amount of MMC-D_{cat} associated with hepatocytes increased as the molecular weight of the dextran chain increased. Interestingly, after i.v. administration, MMC-D_{cat} disappeared from the circulating blood faster than MMC-D_{an} as it rapidly accumulated in the liver through adsorption onto the hepatocytes. MMC-D_{an} on the contrary circulates in the body without any significant interaction in the tissues and slowly accumulates in the liver. By using this

long circulatory half-life, MMC-D_{cat} conjugates may find a future use in systemic targeting to tumour tissue which has capillaries of enhanced permeability to macromolecules (92). The rapid removal of MMC-D_{cat} to the liver, on the other hand, may be a possible targeting device to the liver. However this effect certainly negates it's systemic use. On the other hand, previous studies (93) have shown that local injections of MMC-D_{cat} are suitable for intratumour injection as the conjugate acts as a depot which gradually supplies active drug. This interaction is mediated through electrostatic forces and results in a larger antitumour effect than free MMC-D (94). In this circumstance, any MMC-D_{cat} leaking from the injection site to the circulating system might be rapidly cleared by the liver, thereby reducing systemic side effects.

The long term probability is that polysaccharides will continue to be used in site-specific drug delivery as carriers, and as components to alter distribution *in vivo*. To date, however, only a few derivatives have reached the clinical stage of testing.

1.7.3. Deoxyribonucleic Acid (DNA).

Trouet (53) and Atassi (95) have made extensive use of deoxyribonucleic acid (DNA) as a carrier for several anti-tumour drugs, most often the anthracycline derivatives, daunorubicin and adriamycin. These drugs show a high affinity for DNA without resorting to chemical modifications, and in addition they are resistant to hydrolysis by the lysosomal enzymes. Trouet (53) and other workers (96) demonstrated a number of important advantages when compared to the free drug. Perhaps the most significant is the greatly reduced cardiotoxicity of the complex when compared to the free drug. This was attributed to a lack of endocytic activity shown by the myocardium, and the confinement of the conjugate to specific compartments by capillary barriers. While this is an important advantage there is no indication that DNA enhances the anti-tumour activity of the drugs

themselves. However, both daunorubicin and adriamycin conjugates are more effective antitumour agents in the murine leukaemia, L1210, than the free drug (97). One theory suggests that DNA can augment the pinocytic uptake of a cell (87) and thereby introduce an increased amount of drug to the cell interior. One disadvantage of this could be the rapid clearance of the conjugate by the potent endocytic cells of the RES, resulting in damage to non-target tissue. In a later paper (98), Trouet demonstrated a rapid dissociation of daunorubicin and high molecular weight DNA, indicating the instability of the DNA-DNR bond *in vivo*. The susceptibility of the DNR-DNA linkage may be related to the non-covalent nature of the bond.

1.8. Synthetic Polymers.

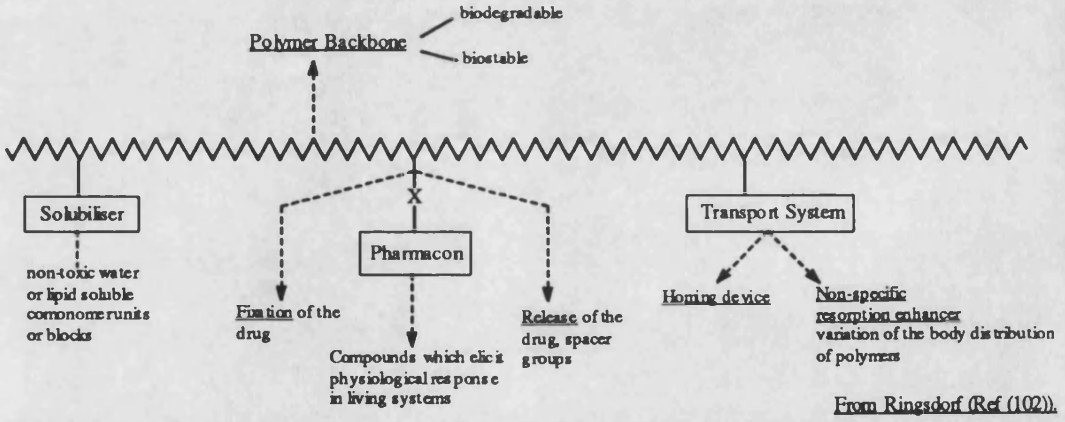
The potential for biocompatible water soluble synthetic polymers is well established (99, 100, 101). Synthetic polymers have been used for a wide variety of biomedical uses including heart valves, polyester arteries, hip-joints and carriers or depots for bioactive agents. Many recent polymeric innovations in the field of drug delivery have followed in the wake of the developments within these specialised fields. The discussion in this section is limited to the water soluble polymeric drug carriers, their fate *in vivo* and their potential use as site-selective, biodegradable drug carriers.

Polymeric-based drug delivery has benefited from a renaissance within the polymer chemistry field which suggests synthetic drug-carriers have a very real potential. Of course, the general characteristics which govern tumour localisation of natural macromolecular carriers also relate directly to the design of synthetic polymeric carriers but the latter group have the enormous advantage that their structure can be tailor made. Manipulation in the laboratory offers an unlimited spectrum of novel polymeric systems where the molecular weight can be optimised and pendant drugs or targeting agents easily added. A corollary of

this is that man-made synthetic polymers are frequently non-degradable and the polymer must therefore incorporate degradable linkages, or be restricted to conjugates of molecular weight lower than the renal threshold, allowing excretion.

A discussion of soluble polymers as drug carriers would not be complete without mentioning the work of Ringsdorf in the mid 1970's. His schematic model for polymeric drugs [*see Figure 1.3*] depicts the design of an optimal polymeric system (102). He suggested that an ideal polymeric carrier would be hydrophilic to ensure water solubility (indeed many effective carriers have been shown to solubilise poorly water soluble anti-tumour agents) and also contain the functional groups necessary to permit covalent linkage to a drug.

Fig: 1.3- Model for pharmacologically active polymers.



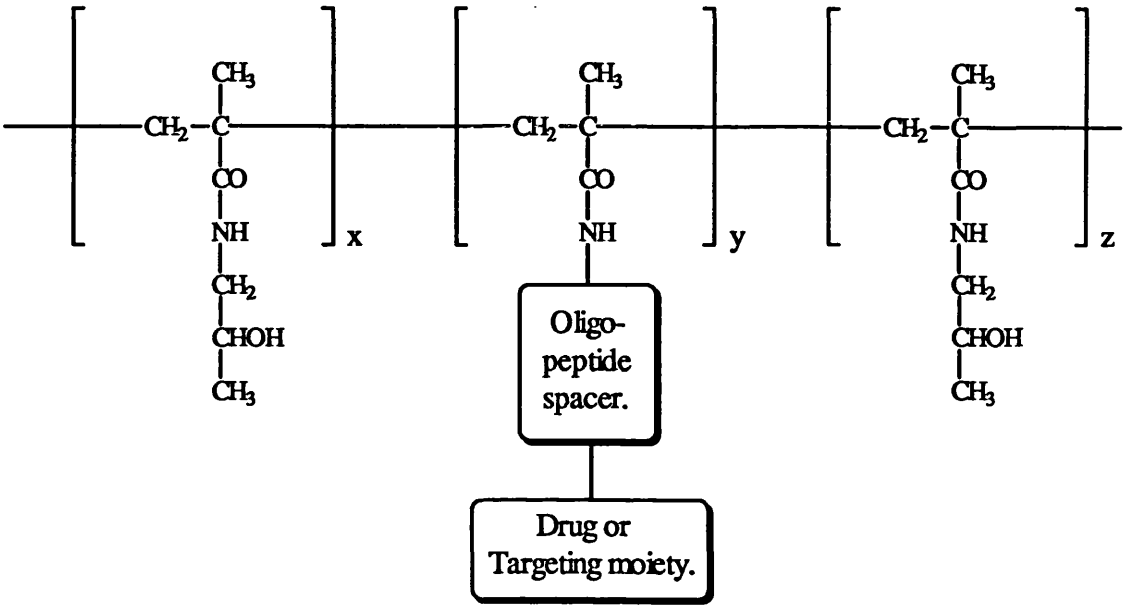
From Ringsdorf (Ref (102)).

The main advantage of biocompatible, water soluble synthetic polymers compared to other drug delivery systems is their targetability. Soluble macromolecules are handled by the body quite differently than particulate carriers (103). Attaching drugs to water soluble macromolecules limits their cellular capture to the pinocytic route and renders them lysosomotropic (*see Section 1.9*).

Of the synthetic polymeric carriers described in the literature, N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers, monomethoxypolyethylene glycol (M-PEG), styrene-co-maleic acid / anhydride (SMA), poly-L-lysine (PLL), poly-vinyl alcohol (PVA) and divinylether maleic anhydride (DIVEMA) have been most extensively explored. The majority of polymers are not chemically inert and can exhibit properties which can be toxic, therapeutic or anti-neoplastic (104). One polymer that is inert and has been systematically developed for the controlled release and targeting of anti-tumour agents (105, 106, 107) is N-(2-hydroxypropyl)methacrylamide (HPMA). HPMA is a biocompatible polymer originally developed as a plasma expander. Copolymers of approximately 20 000 Da size possess characteristics that would optimise it's use as a drug carrier. Conjugates of this molecular weight are relatively small, (*e.g.* polymer-ADR has a diameter of about 8 nm), allowing maximum tumour penetration and effective excretion from the body. Conjugate synthesis is described elsewhere (108), but the method of preparation usually involves initial synthesis of a reactive polymeric precursor by radical co-polymerisation of HPMA with *p*-nitrophenyl esters of N-methacryloylated oligopeptides. These copolymers react readily with compounds which contain an aliphatic amino group in their molecule with the formation of the amide bond, [*see Figure 1.4*].

This versatile concept has generated a number of tailor made conjugates that contain therapeutic agents, *e.g.* adriamycin (109), puromycin (107), daunomycin (107, 110) and melphalan and more recently targetable carriers for photoactivatible drugs (110, 111, 112). The polymer-drug conjugates avoid many of the harmful side effects associated with the use of these drugs. Pharmacokinetic studies show that administration of daunomycin (113) or adriamycin (114) decreases up to 100 fold the level of anthracycline measured in the heart tissue when compared to an equivalent dose of free drug.

Figure 1.4. HPMA. (N-(2-hydroxypropyl)methacyrylamide copolymers.)



Drugs and targeting groups can be bound to HPMA *via* biodegradable oligopeptide side chains which are designed to limit drug release in plasma and serum but be susceptible to degradation by lysosomal proteases (115). Considerable research has been carried out to optimise the side chain length and composition to facilitate degradation by lysosomal enzymes. Pioneering work by Kopecek and his co-workers with HPMA (108) found that Gly-Phe-Leu-Gly showed the greatest susceptibility to lysosomal enzymes. The immunogenicity of the polymers was investigated by determining their ability to induce antibody formation in mice (116). The polymer itself was non-immunogenic, but following repeated administrations of the oligopeptide-drug conjugate a weak, non-significant immunogenic reaction was observed.

Styrene-co-maleic acid neocarzinostatin (SMANCS) consists of two molecules of the synthetic polymer styrene-co-maleic acid / anhydride (SMA) (each of Mw = 1500) [*see*

Figure 1.5(a)] covalently bound to the anti-tumour protein neocarzinostatin (NCS) to give a final molecular weight of approximately 15 000 (9) [*see Figure 1.5(b)*]. The conjugate is soluble in organic solvents and the contrast agent lipiodol used for lymphography. Administration of SMANCS in lipiodol enables both the dose and the tumour image to be quantitated by CT (computer tomography) scanning. In addition it has been shown that extremely efficient tumour targeting can be achieved using this formulation. Polymer conjugation was originally undertaken to increase the plasma half life of NCS (a 10 fold increase was seen) (119) and to improve tumour and lymph node localisation, but additionally the conjugate has been shown to be immunostimulatory (117). In 1983, a clinical study demonstrated that this conjugate has remarkable anti-tumour activity against hepatomas (118). These findings were corroborated by Maeda with rigorous evaluations in 1991.

Figure 1.5(b) describes the chemistry of SMA conjugation with NCS although the reader is directed to a more extensive account by Maeda (119). The SMA contains styrene and maleic acid in which 30 - 50% of the maleic acid is in reactive anhydride form and half of the free carboxyl groups are butylated. This gives substantial hydrophobicity making the conjugate more lipophilic and allowing it to be directed more efficiently to the lymphatics. SMA can react with the free amino groups of NCS, one at Ala-1 and the other at Lys-20.

Figure 1.5(a). Structure of SMANCS (styrene - co-maleic acid / anhydride).

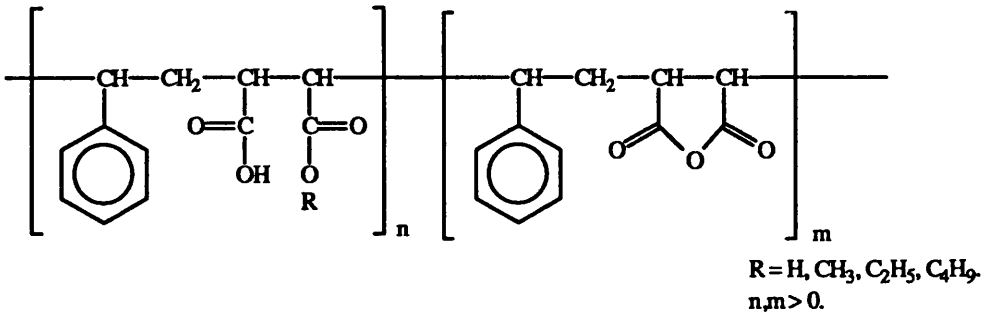
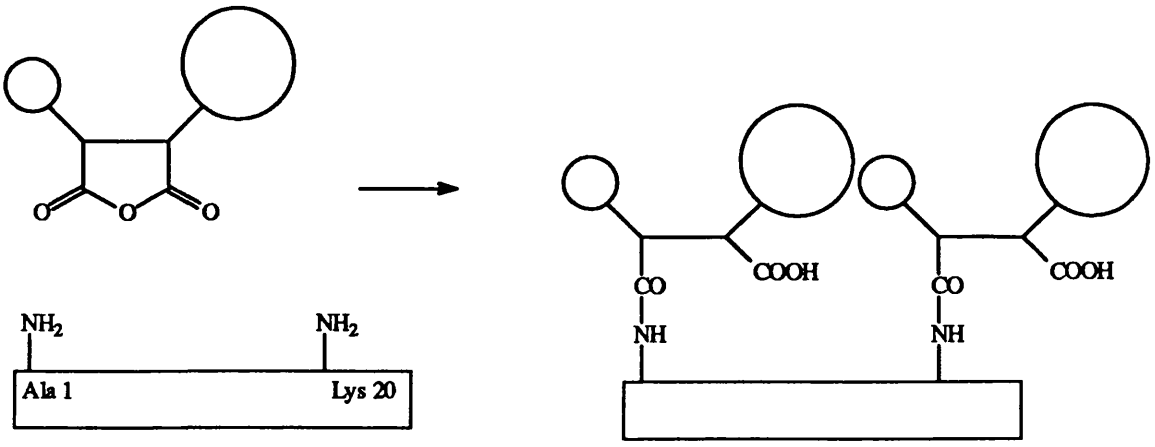


Figure 1.5(b). Diagramatic representation of the reaction with NCS to produce the conjugate SMANCS.



The synthetic polymer divinylether-maleic anhydride copolymer (DIVEMA) [*see Figure 1.6 and 1.7*] shows impressive activity against a wide range of animal tumour models. This activity appears to be mediated by a mixture of cell activation and interferon induction although DIVEMA also gives rise to a range of toxic side effects including anaemia, leucocytosis and hepatosplenomegaly.

DIVEMA (divinyl ether-maleic anhydride copolymer)

Fig. 1.6.

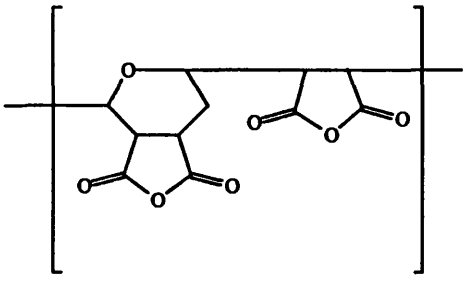
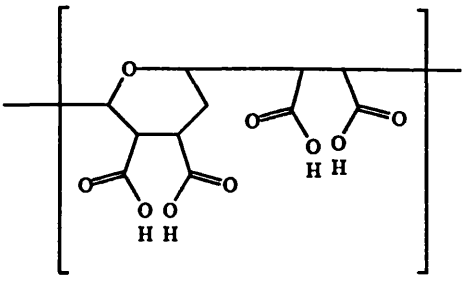


Fig. 1.7.



The chemical structure of DIVEMA:- (*Fig. 1.6.*) unhydrolysed form; (*Fig. 1.7.*) hydrolysed form.

Manipulation of the molecular weight to less than 18 000 D with a narrow polydispersity permitted a reduction in many of the toxic effects without serious impairment of the anti-tumour activity. This modified DIVEMA was termed MVE-2 and has shown promise in the augmentation of the immune system following the immune suppressed period following surgical trauma. This is thought to be a vulnerable period for tumour metastasis which may be alleviated by the administration of MVE-2 (104).

1.9. Drug-carrier conjugates as lysosomotropic agents.

The principle consequence of drug conjugation to a macromolecular carrier is the limitation of the cellular uptake of drug by the mechanism of pinocytosis (120, 53, 102). This is followed by the immediate transfer of the macromolecular drug conjugate to the lysosome, where the majority are thought to exert their activity. Hence they are described as lysosomotropic agents, a term adopted by de Duve, who proposed the phenomenon could also find application in the area of drug delivery (121). At the cellular level, this contrasts

with the ability of most low molecular weight antitumour agents to enter the cell by transversing (either actively or passively) the cell membrane before exerting their pharmacological effect. This is the single most important factor in determining altered pharmacological activity of all macromolecular drug conjugates, irrespective of their composition.

Pinocytosis is a ubiquitous process which can be divided into three categories, fluid phase (non-specific), adsorptive and receptor-mediated [*see Figure 1.8*]. Fluid phase pinocytosis is a constitutive phenomenon common to all cell types, and is characterised by ongoing membrane invagination to form a vacuole, called a pinosome. This enables the capture of macromolecules, such as PVP, dextran and HPMA, (122) present in the extra cellular fluid. Uptake is poorly efficient and the rate is directly proportional to the extra cellular concentration of the macromolecule.

In the case of adsorptive pinocytosis, the substrate binds to the plasma membrane and is internalised with the pinosome (123). This results in an increased rate of uptake over fluid-phase mediated processes (124). Non-specific adsorptive pinocytosis of macromolecules usually occurs due to hydrophobic or charge-dependent interactions, and is frequently independent of cell type (125, 126). It can be influenced by a number of factors, such as; chemical composition of the macromolecule; molecular size (a large molecule having an increased number of interaction sites per molecule); or by having a positive charge, resulting in ionic interactions with the negatively-charged cell surface. Interactions between macromolecules and soluble serum components may result in large complexes, and an increased uptake (127) which is occasionally referred to as "piggy-back" endocytosis.

In contrast, receptor-mediated pinocytosis can be a highly efficient, highly specific uptake process. The ligand of, for example, low density lipoprotein (LDL), hormone (EGF), or plasma proteins, can occupy a receptor, resulting in internalisation of receptor-ligand

complex. Most endocytic vesicles fuse with and deliver their contents to primary lysosomes, thereby forming secondary lysosomes. In some cells, a part of the endosome escapes degradation and is transferred to another part of the plasma membrane. Here the vesicle fuses and releases it's contents to the exterior, a process called exocytosis. If the cells are polarised, *e.g.* endothelial cells, macromolecules can be transferred from the apical to the basolateral cell membrane, a process called transcytosis.

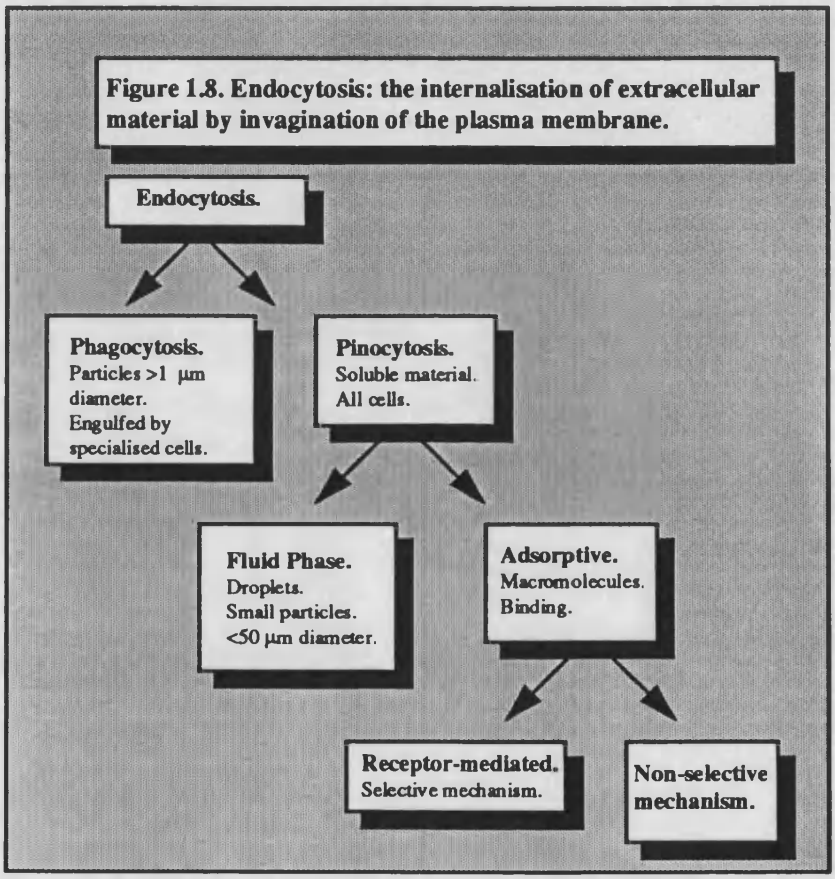


Figure 1.8. Endocytosis: the internalisation of extracellular material by invagination of the plasma membrane.

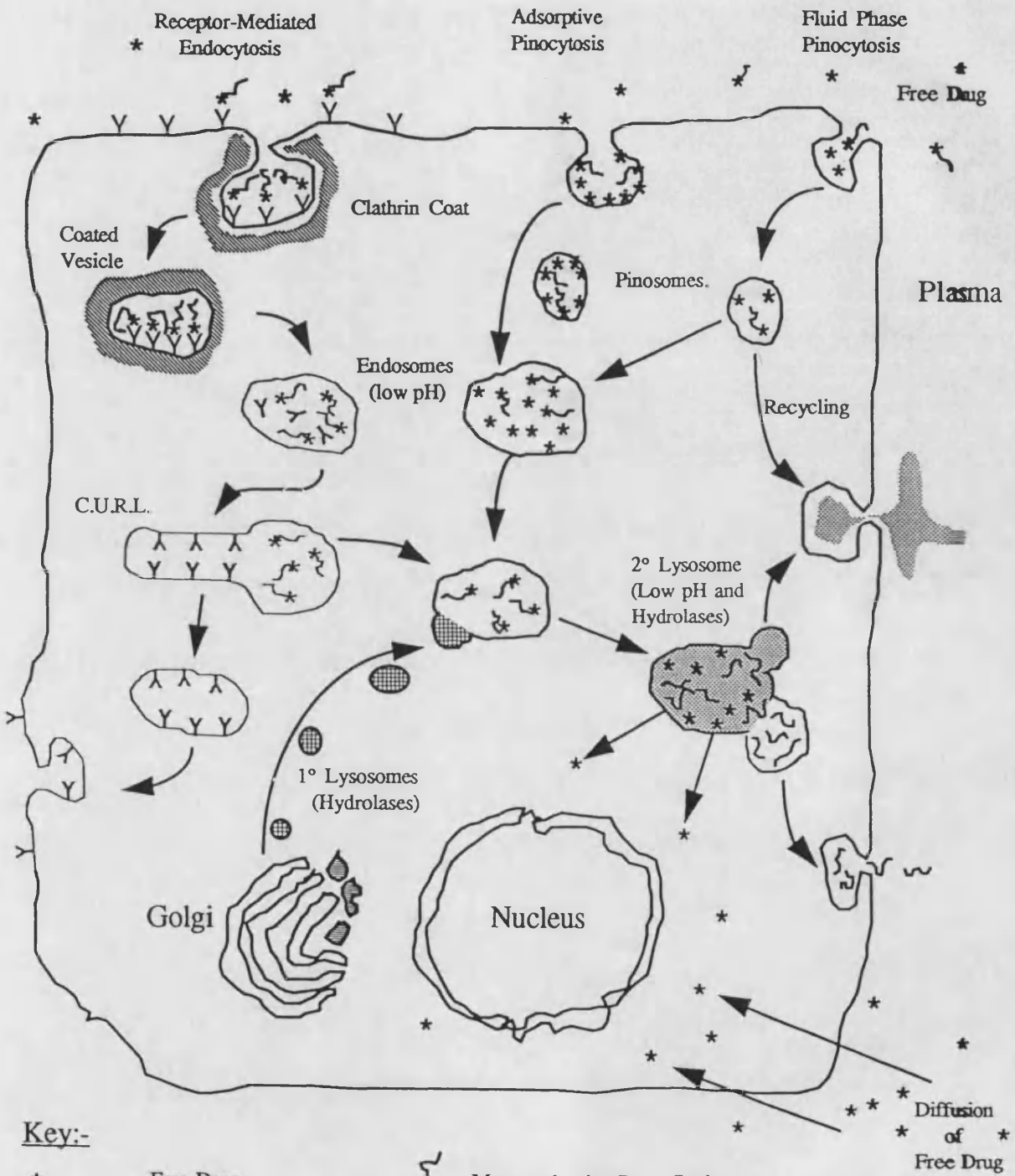
In the case of receptor-mediated endocytosis, the receptor-ligand complexes tend to cluster into special membrane areas that are invaginated forming coated pits. The number of available receptors on the cell surface is therefore temporarily reduced (down regulated) for some time after the uptake (128). This down regulation is a way in which the cell can control the uptake of specific macromolecules and has limited the use of monoclonal

antibodies to act as drug carriers (129). The coated pit pinches off from the cell surface and rapidly loses it's clathrin coat before fusing with other vesicles to form large endosomes. The endosome (or C.U.R.L. - Compartment of Uncoupling of Receptor and Ligand) has a dual purpose. An active proton pump maintains a low pH and enables the receptor to dissociate from the ligand (130). Often the receptors are recycled to the plasma membrane, *e.g.* LDL and asialoglycoprotein (ASGP) receptors (131), or in contrast, the receptor can be transferred to the lysosome with the ligand, *e.g.* epidermal growth factor. Most macromolecules are then directed *via* a series of vesicle fusion events, which may involve the Golgi apparatus, into a secondary lysosomal compartment where they are exposed to lysosomal enzymes. A proportion of the endosomes may fuse with the Golgi apparatus which can rapidly transfer ligands into primary lysosomes and recycle receptors to the plasma membrane (132). The acid milieu of the lysosomes comprises approximately sixty enzymes including proteases, nucleases, phosphatases, glycosidases, sulphatases, lipases and phospholipases, and is capable of degrading every class of natural macromolecule entering the cell (133). Within the lysosome, the macromolecules are degraded to small metabolites which can be transported across the plasma membrane to be used in metabolism in other parts of the cell [*see Figure 1.9*].

Macromolecules that are non-biodegradable are retained within the lysosome for long periods of time, for example, hydroxyethyl starch and PVP have a half-lives of 132 and 64 days respectively in the liver and spleen of the rat (134) while the lysosomal half-life of the endocytosed protein is, in general, only a few hours (135). This means that a drug carrier that is metabolically inert or only slowly biodegradable may reside within secondary lysosomes long after the drug has been released and eliminated. Lysosomal accumulation (thesaurosis) of the carrier may be acceptable, or even positively advantageous, if the target cell is a cancer cell, but less so if the target is a normal cell (136).

Figure 1.9.

Endocytosis: The Uptake Mechanism of Macromolecular Drug Carriers *vs* Free Drug.



Key:-

*	Free Drug.	*	Macromolecular Drug Conjugate.
	Primary Lysosome.	Y	Receptor, eg. LDL receptor.
	Drug Carrier.		C.U.R.L Compartment of Uncoupling Receptor and Ligand. (Late and early endosomes).

Reports suggest that thesaurosis may cause an increase in osmolarity within the compartment which may result in the swelling and rupturing of the lysosomal membrane whereas other studies suggest the storage of non-degradable or slowly degradable material within the lysosome may impair the normal phagocytic capacity of the target cell (137).

In general, the lysosomal membrane is impermeable to hydrophilic compounds that are larger than the natural monomers used in the various metabolic pathways, *e.g.* amino acids, monosaccharides (136). In order to avoid "storage disorders" and effect the complete removal of a carrier from the body, the carrier must therefore be totally biodegradable within the lysosome. The rate of degradation within the lysosome will be dependent on the hydrophilicity of the carrier, the type of linkage between the carrier and drug, and the degree of drug substitution. Lysosomal storage disorders will also depend on the dose given, the frequency of administration and the kinetics of lysosomal accumulation.

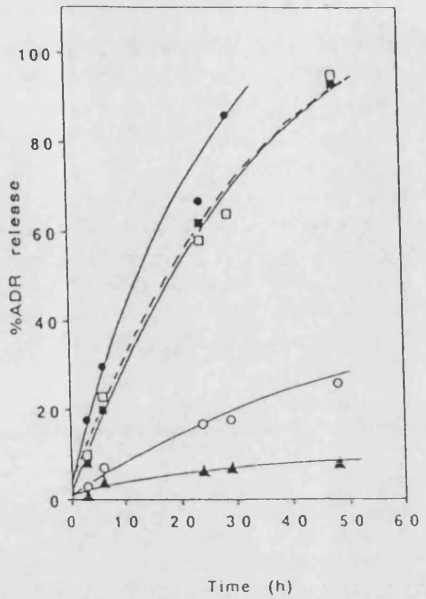
1.10. The incorporation of biodegradable spacer molecules in drug-carrier conjugates.

Ringsdorf (102) conceived the concept that covalent conjugation of a drug to a polymeric carrier *via* a biodegradable spacer amenable to specific enzymatic or hydrolytic cleavage would be advantageous. Careful choice of the drug-polymer linkage can ensure that the drug is released only following internalisation and subsequent cleavage by lysosomal enzymes. The conjugate should be completely stable and inactive in the bloodstream. If the spacer is cleaved before arrival at the target site, the carrier becomes totally redundant , and the drug is released, which may cause harmful side effects. In early studies, mixtures of isolated rat lysosomal enzymes (tritosomes) were used as the most convenient *in vitro* test system to study spacer degradation. Later, purified bovine spleen enzymes, cathepsins B, H and L were also used, which have activity analogous to human cathepsin (138).

The acidic pH of some solid tumours, albeit an extracellular phenomenon, suggests that an acid-sensitive linker may provide both rate control of drug delivery and some tumour selectivity. Shen and Ryser (139) were the first to demonstrate the use of pH-sensitive spacers to conjugate daunorubicin to poly(lysine), using the pH-sensitive *cis*-aconityl spacer. The spacer had a half-life of less than 3 hours at pH 4, but was much more stable at pH 6, having a half-life of more than 96 hours.

Peptidyl spacers have been developed for attachment of drugs to polymers as their amino acid sequence can be specifically chosen to facilitate intracellular degradation by the lysosomal proteases (138, 140). Addition of the thiol-proteinase inhibitor leupeptin to the incubation medium caused a pronounced decrease in the rate of intracellular hydrolysis of peptidyl side chains indicating the involvement of thiol proteinases in degradation (141). These include the lysosomal thiol-dependent proteases, cathepsins B, H, L (142) and lysosomal aspartic protease cathepsin D (143). Cathepsin D has a pH optimum in the acidic range (pH 2.8 - 5.0), whereas the thiol-dependent enzymes have much broader optima and retain considerable activity at neutral pH. Drug conjugates that contain non-biodegradable spacers do not show anti-tumour activity *in vivo* confirming the need for extensive research to find a degradable peptide link that will ensure efficient drug release once the carrier is within the lysosomal environment. The first drug bound to a non-degradable polymer by enzymatically-degradable side chain was prepared by Jatzkewitz (145). He used poly(vinyl-pyrrolidone)(PVP) as a carrier for 3,4,5-trimethoxyphenethylamine (mescaline) which was attached to the carboxylic acid of the dipeptide spacer, glycyl-L-leucine, as an amide.

Figure 1.10. Time course of enzymatic hydrolysis of HPMA polymeric prodrugs containing adriamycin (ADR) by tritosomes.



(O) Gly-Leu-Gly; (▲) Gly-Phe-Gly; (❑) Gly-Phe-Leu-Gly; (■) Gly-Phe-Leu-Gly (polymer also containing galactosamine); (●) Gly-Leu-Phe-Gly. [from Subr *et al* (144)].

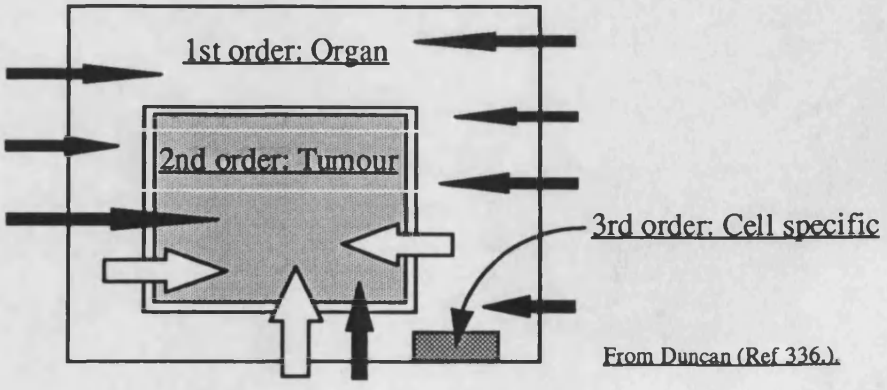
Kopecek (146) later studied a series of co-polymers of HPMA bearing oligopeptide side chains terminating in 4-nitroaniline (drug model). The study convincingly showed that by changing the length and composition of the oligopeptide sequence it was possible to change the rate of drug model release. The highest drug release was obtained with cathepsin B-catalysed hydrolysis of the oligopeptide sequences, Gly-Phe-Tyr-Ala and Gly-Phe-Leu-Gly. Similar results were reported by Subr *et al* (144) who found that the rate of release of melphalan and adriamycin from HPMA copolymers varied from 10% (Gly-Gly) released per day to more than 80% (Gly-Phe-Leu-Gly) per day [*see Figure 1.10*]. Trouet (98) in 1984 used a variety of spacer arms based on leucine and alanine to link albumin to the anthracycline, daunorubicin.

The most successful of those conjugates were based on the tripeptide, Leu-Ala-Leu-daunorubicin or the tetrapeptide, Ala-Leu-Ala-Leu-daunorubicin, which showed greatly improved chemotherapeutic activity against L1210 leukaemia both *in vitro* and *in vivo*. Several amino acid and dipeptide derivatives of daunorubicin were also synthesised with Leu-daunorubicin, Leu-Leu-daunorubicin, and Ala-Leu-daunorubicin, showing striking activity against sub-cutaneously implanted L1210. The hydrophobic nature of these conjugates suggests that they may easily gain access to the sub-cutaneous tissues and that they are extensively hydrolysed into daunorubicin by lysosomal enzymes present in the tumour cells.

1.11. Targeting.

Many drugs show little, if any, inherent specificity for their site of action. Selective targeting of drugs to a specific site will not only reduce the side effects to the host, but will also increase the therapeutic efficacy. Many first order delivery systems [*see Figure 1.11*] have been identified that achieve very effective organ (compartmental) drug targeting.

Figure 1.11.: Theoretical aspects of first- and second-order targeting.



From Duncan (Ref 336.).

Liposomes, particulate systems and macromolecular carriers have been developed which can deposit a large percentage of an intravenous dose into the liver, lung or

kidneys. This approach is advantageous for regional delivery of antitumour agents for treatment of primary or secondary disease, but clinical success is awaited. To achieve tumour-specific (second order) targeting, it is necessary to identify unique features of tumour cell biology that will concentrate drug within the tumour. Researchers have investigated both passive and active targeting strategies to achieve this goal.

1.11.1. Passive targeting.

Passive targeting is often associated with liposomal and particulate fields of drug delivery with their embolisation or capture by phagocytic cells of the reticulo-endothelial system. However, there is unequivocal evidence that many macromolecules do passively accumulate within solid tumours and it is unlikely that this is solely as a result of enhanced pinocytic capture. The phenomenon has been studied in depth by Maeda and co-workers (148) who termed tumour accumulation of macromolecules the "enhanced permeability and retention effect (EPR)", attributing it to two factors; leaking tumour vasculature and lack of effective lymphatic drainage.

The capillary endothelium is of profound significance in the biodistribution of macromolecules and drug conjugates. The three main types of capillary endothelium are; continuous, with the lowest permeability characteristics, found in the skeletal muscle, skin, lung, heart, brain; fenestrated, present in the kidney, gastro-intestinal tract mucosa and endocrine glands; and sinusoidal found in the liver, spleen and bone marrow. Fenestrated and sinusoidal epithelium are virtually freely permeable to molecules < 20 KDa although most tumour capillaries are of a continuous type (149). It has been demonstrated that some tumours, particularly those found in the liver, kidneys or exocrine glands, can induce the angiogenesis of fenestrated or sinusoidal vessels (150). A substantial number of solid tumours have been reported to possess blood vessels that are relatively permeable to macromolecules, compared with blood vessels serving normal tissue (151, 152). This leakiness was shown to be confined to the venules running along

the periphery of tumours and at the tumour - host interface (153). This phenomenon has been attributed to tumour angiogenesis, *i.e.* the process by which tumours attract in growths of new capillaries to provide a blood supply to neoplastic tissues. Such in growths are almost entirely composed of endothelial cells, whereas normal capillaries contain pericytes. The function of pericytes is to induce maturity and differentiation to the capillary, thereby reducing non-specific permeability.

The EPR effect can also be augmented by a tumour vascular permeability factor (154) and other factors such as bradykinin and it's derivative (3-hydroxypropyl)-bradykinin. Kinin is a potent permeability and pain-inducing factor, with highly elevated levels shown to be present in rodent and human ascitic fluid of many cancers (155). In addition, it has been demonstrated that a vascular permeability factor is secreted by a broad spectrum of animal and human tumour cells (154). The physiological purpose underlying hyperpermeability of tumour epithelia is concerned with the import of sufficient fibrin to permit the formation of a functional tumour interstitium, essential to successful tumour development. This clinically-observed phenomenon (156, 157) allows the passive targeting of soluble macromolecules through enhanced tumour extravasation. The process is generally size exclusive (< 30 nm diameter) and therefore amenable to soluble macromolecules, whose functional size is often less than 30 nm (typically 5-8 nm) (158). There is little morphological evidence to date suggesting the increased rates of extravasation observed are due to enlarged interendothelial spaces. Instead, authors suggest that the process is mediated by enhanced vesicular transport or by transendothelial channels (159).

Lipophilic molecules and macromolecules ordinarily show little bulk diffusion into the blood circulation following extravasation from normal tissue. Instead they are preferentially returned to the general circulation *via* the lymphatics (160). In solid tumours, this situation is fundamentally different since they lack any organised system of lymphatic drainage [*see Figure 1.12*]. Consequently, components of the interstitial fluid

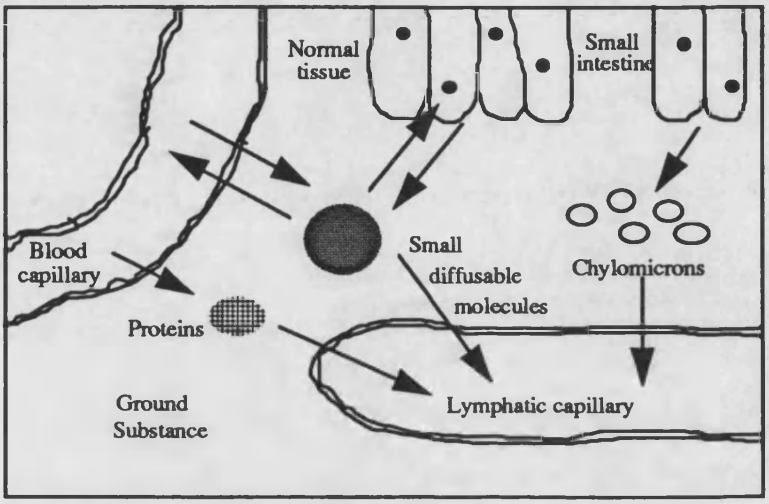
that are not drained by entry into the post-capillary venules, have no effective mode of drainage and accumulate. This was demonstrated by using the lipid lymphographic agent, lipiodol (161). This may give rise to elevated interstitial pressures which encourages the movement of macromolecules through the tumour interstitium by a process called convection. After seeping through the tumour, conjugates will collect on the periphery of the tumour, from here they are drained by the lymphatics of normal tissue. This simple concept forms the basis of the EPR effect, and is the basic mechanism being applied in cancer-targeting chemotherapy.

Angiotensin II-induced hypertension has been proved to result in the selective increase in the blood flow in tumour tissue only, and not in normal tissue (162). Therefore, following angiotensin II-induced hypertensive state, anticancer agents bound to macromolecules leak out less effectively into normal tissues. Conversely, the lack of vascular contraction within the tumour bed facilitates leakage of plasma and macromolecules which may enhance the selective deposition of drugs. This has recently been effectively demonstrated *in vivo* with SMANCS and (^{51}Cr)BSA administration to rats (163). In addition, anticancer drugs would be more readily confined to the normal circulation following angiotensin II administration, reducing the side effects seen using an equal amount of anticancer agent. It was noted that small molecules, *i.e.* (^{3}H) methyl glucose were able to diffuse freely in and out of the blood vessels and showed no preferential accumulation Thus, both the anatomical differences and the molecular size and property of the drug appear to be critical for selective tumour targeting.

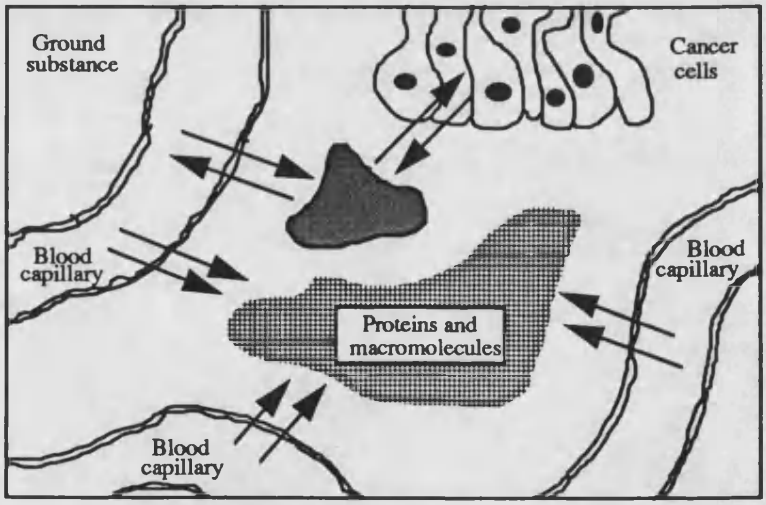
Although drug carriers can display effective first order targeting to the organs, *e.g.* liver, and advantageous pharmacokinetics with ability to concentrate passively in solid tumours, the feasibility of tumour-specific second order (active) targeting is continually being pursued. The use of carriers that recognise an antigen receptor or some other marker at the target site may enhance this delivery of drugs. In recent years, much progress has been made in efforts to target cytotoxic agents to tumour cells using

monoclonal antibodies recognising tumour cell-associated antigenic markers (129, 164) [*see Section 1.7.1.1.*].

Figure 1.12. Schematic representation of extravasation and fluid drainage in normal tissue and tumour tissue.



(A) Normal tissue:- (intact endothelium, lymphatic drainage).



(B) Tumour tissue:- the EPR effect (hypervascularity, no lymphatic capillary, limited lymphatic drainage).

Ref; Maeda *et al* (148).

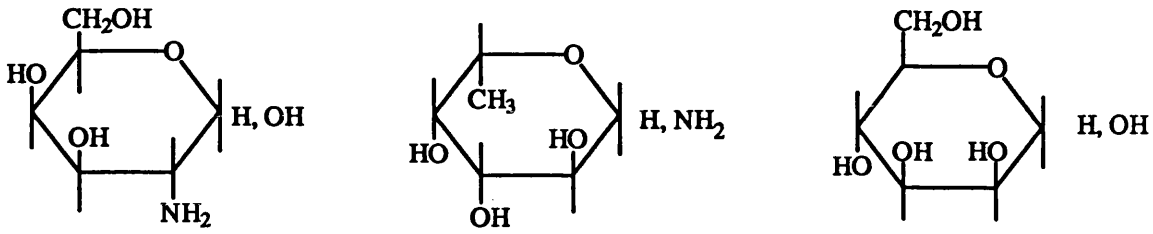
1.11.2. Active targeting.

A number of ligands of importance to metabolism are brought into the cell by receptor-mediated endocytosis and include nutrients such as vitamin B_{12} (as a B_{12}-transcobalamin

II complex), and growth factors such as insulin. This highly efficient uptake process does have the potential for tumour targeting when cells display a high density of ligand-specific membrane receptors. Unfortunately, many of these receptors have a broad cellular distribution, *e.g.* transferrin, LDL, epidermal growth factor, making them less than ideal targets. Recognition systems for terminal sugars on carbohydrate chains of glycoproteins include; galactose receptors of mammalian hepatocytes (165), Kupffer cells (166), liver endothelial cells (166) and bone marrow (167), the N-acetyl glucosamine receptor of avian hepatocytes (168), the mannose-6-phosphate receptor on a variety of mammalian tissues (169), the receptor for a high affinity for fucose on mouse L1210 leukaemia cells (170) and the mannose/N-acetyl glucosamine receptor on alveolar macrophages (60) and other cells, including the reticulo-endothelial system [*see Figure 1.13*]. The asialoglycoprotein receptor is exclusive to hepatocytes and responsible for the clearance of galactose-terminated glycoproteins from the circulation (171). This receptor has been used to good effect. In 1986, it was shown that HPMA co-polymers modified with galactosamine were internalised by rat liver hepatocytes, *in vivo*, and a drug was liberated intralysosomally (112). When administered i.v. at low dose, the polymer ADR-Gal is captured very effectively by the liver (more than 80% of the dose administered). However, receptor saturation does occur at higher doses and thus significantly decreases the desired specificity (105). Therefore, to facilitate first order targeting to the liver for treatment of primary or secondary liver disease, a continuous infusion or repeated low bolus dose regimen would be ideal.

The hepatic galactose receptors occupy a dominant role in the clearance of galactose-bearing conjugates. This is advantageous to site-selective drug delivery but it is possible that other potentially useful or harmful galactose mediated interactions may be obscured by the hepatic recognition systems. Seymour *et al* (172) demonstrated that both intraperitoneal and sub-cutaneous injection of HPMA co-polymers, bearing a high concentration of galactose residues, resulted in pronounced accumulation in local fatty tissue.

Figure 1.13 Targeting moieties.



(a). D-galactosamine. (b). L-fucosylamine. (c). Mannose.

Optimistically, this interaction may afford the possibility of drug targeting to fat cells, although it underlines the dangers of targeting drug carriers to supposedly discrete second order receptors.

Seymour *et al* reported, in 1987, enhanced deposition of HPMA copolymers bearing mannosamine or glucosamine in liver macrophages of rats. These *in vivo* experiments demonstrated that polymers bearing the targeting moiety were internalised rapidly by a common receptor. This interaction could be inhibited by free D-mannose or L-fucose, giving further evidence for a receptor-mediated system (173). Bard *et al* (174) proposed a different approach to the targeting problem. A superactive analogue of α-melanocyte stimulating hormone (MSH) was conjugated to diethylenetriamine penta-acetic acid (DTPA) (MSH(4-10)-DTPA), and was successfully used for the targeting of imaging agents for mice melanomas *in vivo*. Recently, Sunassee *et al* (175) produced ^{125}I-labelled MSH-HPMA polymer conjugates which showed a broad body distribution in normal mice. Unfortunately, preliminary experiments using mice bearing a MeWo (melanoma cell line) xenograph, suggested that less than 1% of an intravenous dose localised in the tumour.

Photodynamic therapy (PDT) has been developed in order to achieve double targeting; site specificity of the drug by virtue of a targeting moiety (176, 177) and light specificity. Photosensitizers, *e.g.* chlorin e_6 bound to targeted HPMA co-polymers will become associated with target tissues in discrete areas of the body. However, by illumination of the target area only two-fold selectivity may be achieved and side effects reduced. Preliminary results are satisfactory although one major side effect is phototoxicity which requires patients to remain out of direct sunlight for six to eight weeks following treatment.

It is perhaps obvious to state that targeted polymer drugs enter the cell *via* different receptor mechanisms, depending on the targeting moiety. In the case of the HPMA-chlorin e_6 targeting groups, galactosamine and anti-Thy 1.2 antibody, this gave rise to differences in the observed phototoxicity (176). The asialoglycoprotein receptor participates in the regulation of serum glycoprotein homeostasis (178), while Thy 2.1 antigen may function as a signal transducing molecule (179). Such differences in phototoxicity may be explained by; (a) the different number of membrane receptors; (b) the difference in the affinity of the receptors; (c) different rates of endocytic uptake; (d) different endosomal fates.

1.12. Macromolecular Drugs and Enzymes.

Most pharmacologically active agents are low molecular weight compounds which readily penetrate into all cell types, but are also rapidly excreted. Consequently large and repeated doses must be applied in order to maintain a therapeutic effect. However, due to a limited specificity of the majority of drugs a range of serious side effects are often observed. By conjugating a drug to a soluble polymeric macromolecule such side effects are often reduced as the conjugate uptake is restricted to certain target

cells. At the same time the efficiency of drug action can be dramatically increased by directing them selectively to their cellular targets.

One of the most important limiting factors when designing a polymeric drug delivery system is the drug carrying capacity of the conjugate. The drug must possess adequate functional groups for conjugation but these should be separate from the active site of the drug. The pharmokinetics, formulation and clinical issues should also be addressed, together with the influence of high drug substitution on the pattern of biodistribution and biodegradation. McCormick (180) demonstrated that metribuzin when linked to PVA *via* a number of different linkages exhibited an increased drug release when the degree of substitution was decreased. It was proposed that at high drug substitution the access of water due to the hydrophobicity of the drug was restricted and this was responsible for the rate limiting step of degradation.

Although many anti-tumour agents have been conjugated to soluble macromolecular drug carriers, three classes of drug have received most attention:
(a) Anti-metabolites:- drugs in this category are analogues of essential endogenous compounds, (for example replacing -OH and -H in folic acid with $-NH_2$ and $-CH_3$ respectively produces methotrexate (MTX)). MTX [*see Figure 1.14(a)*] inhibits the enzyme dihydrofolate reductase in both tumour cells and normal tissues. Although potent effects on tumour cell growth can be obtained toxicity against other rapidly dividing cells makes it's effective use difficult;
(b) Drugs affecting DNA:- the anthracycline antibiotics, *e.g.* daunomycin, adriamycin a similar problem is encountered with intercalating agents which are effective antitumour agents against a variety of human malignancies. Daunomycin is principally active against acute lymphocytic leukaemia (ALL), but adriamycin has a much broader spectrum of activity against a variety of tumours including certain solid tumours [*see Figure 1.14(b)*]. This class of drugs is believed to inhibit the activity of DNA and RNA polymerases. However, in addition to causing bone marrow depression these drugs demonstrate

serious and irreversible cardiac toxicity which frequently necessitates withdrawal of therapy (181). Analogues of the anthracyclines such as epirubicin and idarubicin have improved properties but their clinical success has been slight and adriamycin is still frequently used;

(c) Alkylating agents:- *e.g.* melphalan. All effective anti-neoplastic drugs in this class possess two alkylating groups [*see cis-platin, Figure 1.14(c)*]. The highly reactive cyclic cations formed spontaneously in an aqueous solution bind to side chains of large molecules, especially the guanine codon of DNA. This causes functional damage to the DNA. Any unbound cyclic cation is spontaneously hydrolysed to an inactive alcohol. Cis-platin has a wide spectrum of clinical activity but is often associated with nephrotoxicity and emesis. Chlorambucil is another derivative of Nitrogen mustard which has been cross-linked to a number of carrier molecules, *e.g.* TS-mAb (3, 182).

Neoplastic drugs which are routinely administered to patients in high doses such as 5-fluoruracil and MTX are poor candidates for use with polymeric carriers considering the paucity of conjugates finally reaching the tumour (183). For this reason many investigators have chosen to combine more potent drugs, *e.g.* anthracyclines, mitomycin C and cis-platin analogues to the polymer.

The natural toxins are ideal in this respect since they are powerful therapeutic agents, examples include ricin, diphtheria toxin and abrin. Unlike drugs which usually react stoichiometrically, plant toxins react catalytically and are thereby capable of extreme cytotoxicity in very low doses; one molecule of toxin per cell may cause cell death (184). These toxins comprise of two polypeptide chains (A and B, disulphide bridged, each about 30 KD) neither of which is separately toxic to cells (185). The B-chain is concerned with binding to the cell surface and internalisation of the A-chain into the cytosol where it terminates protein synthesis (186). The toxins have been conjugated to a number of macromolecular carriers in an attempt to restrict their action to specific locations, these include antibodies (187), lectins (188), hormones (189) and epidermal

growth factor (190). It is usual to use the A-chain alone since the complete molecule may still retain some non-specific action leading to toxic effects on normal cells. Certain polymeric biological carriers (tumour specific mAb) or biological response modifiers (*e.g.* recombinant inter-leukin-2) are capable of exerting a direct cytotoxic or an immune-system mediated effect respectively. Conjugation of rIL-2 to PEG markedly increased the anti-tumour potency of rIL-2 in the Meth A sarcoma model (191). Synthetic polymers with antineoplastic activity have also been reported (104).

Figure 1.14(a). Methotrexate.

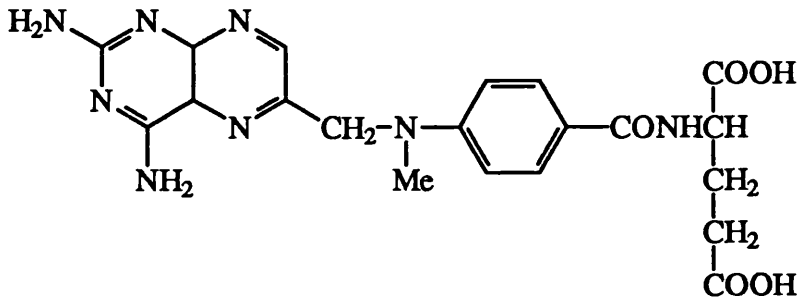
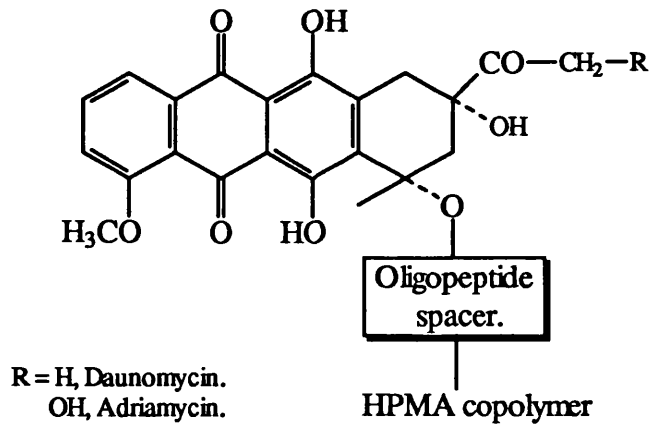
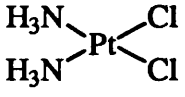


Figure 1.14(b). The Anthracyclines.



R = H, Daunomycin.
OH, Adriamycin.

Figure 1.14(c). Cis-Platin.



Photosensitisers are molecules that are activated by visible light and are currently being assessed as cytotoxic agents both for cancer therapy and for autoagressive immune disorders (176, 177). Two photosensitisers that have been extensively studied are the porphyrins and chlorins [*see Figure 1.15*], with the latter achieving greater tissue penetration and photoactivation. To this end, contemporary interest now centres on photosensitisers activatable by red light which is of a higher wavelength and enables a better tissue penetration. Photodynamic therapy (PDT) is based on the dye-sensitised photo-oxidation of intracellular components with damage at multiple sites. The photolytic activity of galactosamine targeted or anti-Thy 1.2 targeted polymeric (HPMA) chlorin e_6 has been documented, with promising results. However, more work is needed to evaluate their full therapeutic potential.

Conjugating a drug to a soluble polymeric macromolecule will render the drug lysosomotropic. The drug must therefore not be acid labile or undergo lysosomal degradation. Equally if a drug requires access to an intracellular receptor to initiate activity then it is useless to bind that drug to a carrier *via* a non-biodegradable linker, this will prohibit drug release and therefore cytoplasmic penetration. Similarly compounds with limited plasma stability are often not good candidates for polymer conjugation since they would be theoretically inactivated whilst in transit before reaching their cellular target. The use of macromolecules has to some extent resolved this problem as the carrier often acts to sterically protect the drug from hydrolytic degradation increasing the stability of the drug.

There are some drugs which are active even when permanently bound to a carrier. Trypsin-kallikrein inhibitor retains most of it's activity against trypsin when bound to the polyaspartamide carrier (192) provided the lysine residues are not blocked. Another example is novocaine which retained 95% of it's activity when bound to dextran (72). On the contrary, the mitomycin C-dextran [*see Figure 1.16*] conjugates exert their

toxicity following chemical hydrolysis, thus providing a sustained release of the active drug.

Figure 1.15. HPMA copolymer conjugate containing chlorin e_6. *(Ref = Krinnick et al (337)).*

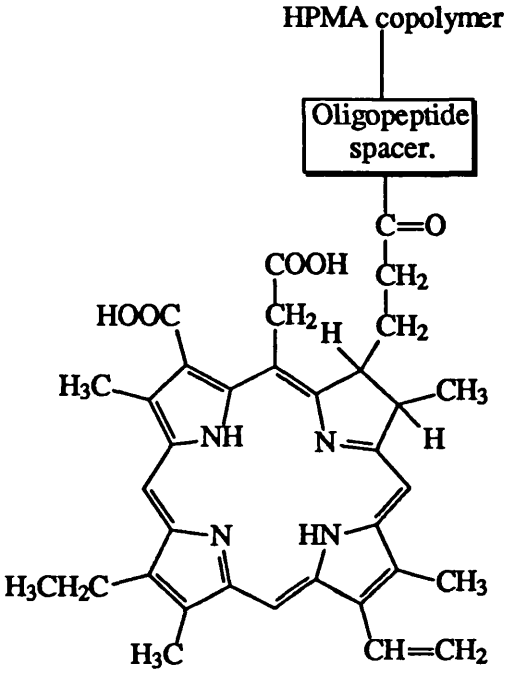
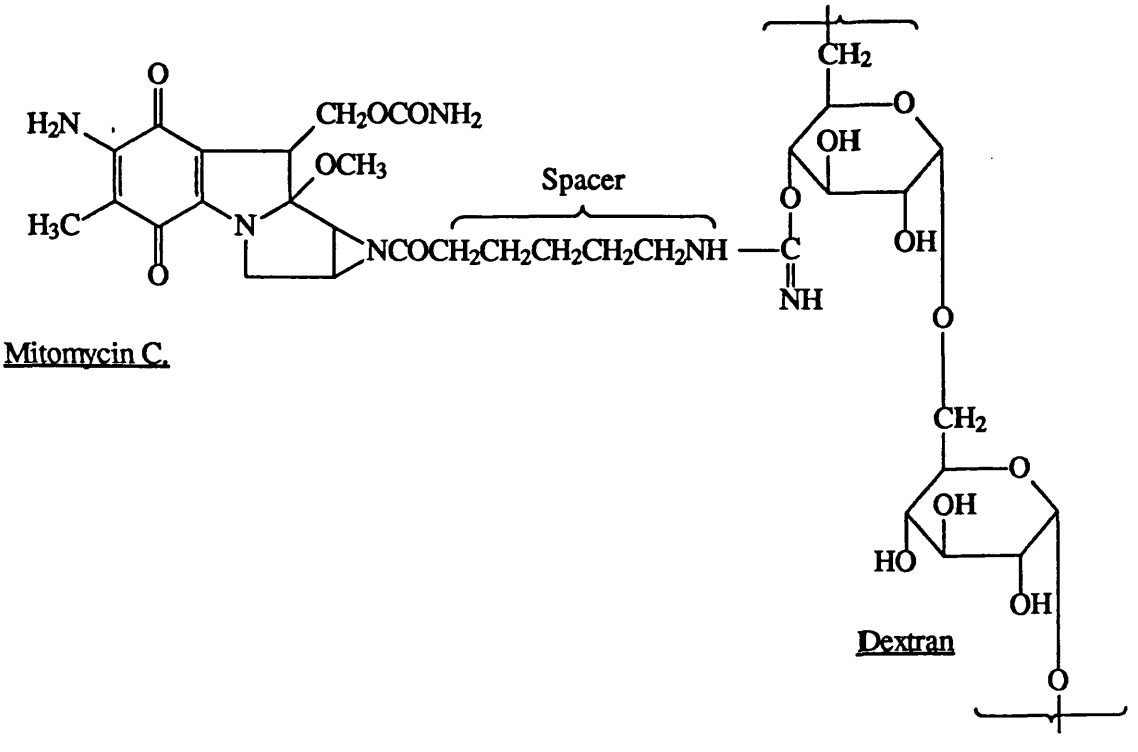


Figure 1.16. Mitomycin C-Dextran Conjugate.



Mitomycin C.

Dextran

1.13. Polyethylene glycol.

One of the most important factors in designing a biodegradable carrier is the choice of the polymer. Existing as the major component within the final product the polymer will influence the pharmacokinetics of the system. With extensive study PEG has found applications as a plasma expander and is approved by the Federal Drug Administration (FDA) for use in drugs (parenterals, topicals, suppositories, nasal sprays), foods and cosmetics. In addition, the linear polymers have been employed as soluble matrices for liquid - phase peptide synthesis, metal complexing and phase partitioning of immiscible aqueous solutions. PEG has as an uncharged, hydrophilic, inert, non-immunogenic nature (193) and exists in a variety of molecular weights. Other useful properties include their low cost, ease of chemical modification and solubility in both aqueous and organic solvents. It is therefore clearly suitable as a drug carrier in the body. Removal of PEG from the body takes place passively through renal clearance, with a higher molecular weight polymer having a greater half-life. The above qualities alone do not constitute a biodegradable carrier compound. However, by activating the two functional groups present on PEG the polymer can be modified to carry groups which are susceptible to degradation.

1.13.1. The Advantages of PEG-Modified Proteins.

The potential value of proteins as therapeutics has been recognised for years. To use as a drug a compound which is found *in vivo* would seem to have no equal. Such a drug would have activity at specific sites only and should not illicit any side effects. However, the therapeutic applications of a number of proteins, peptides, hormones, enzymes and drugs are often compromised. Proteins tended to have low stability as pharmaceuticals and short blood circulating lives. This problem arises whether the source of protein is human, exogenous or recombinant and is often mediated through immunological processes and circulating proteases. In addition to this, repeated

administration of proteins is often associated with the development of hypersensitivity (194, 195, 196) which could lead to anaphylactic reactions and even death. Hypersensitivity is a problem irritated by the need to maintain regular administration, or increase high dosages to combat efficient blood clearance. The clearance of therapeutic drugs *in vivo* results in the characteristic rise and fall of the circulating drug. Often this results in sufficient beneficial levels that are maintained only for a brief period with much of the drug circulating at sub-clinical dosage for the majority of time.

The development of cloning techniques and the establishment of large scale manufacturing processes allowed the production of huge quantities of human recombinant protein (HrP). It was believed that proteins derived from human sources would eliminate many of the problems found with non-human protein. In general the immunogenic problems of protein therapeutics were resolved, although poor stability and short circulating half life still existed. This again made it necessary to administer large doses of what was now a high cost drug due to the recombinant technology required to produce HrP. Non-human protein costs were generally low, and resulted in low cost pharmaceuticals.

To overcome the problems associated with the use of proteins (from both human and non-human sources) for therapeutics, a number of drug delivery technologies are being evaluated. The discussion in the preceding chapter hinted at the plethora of drug delivery molecules under investigation. In some cases proteins can be manipulated with polymers to eliminate some of the drawbacks of native proteins and it is an area of research which is crucial to the development of a general application for enzyme therapy.

Several enzymes have been favourably modified with PEG (196, 197, 198, 199). As a general rule, PEG-modified proteins exhibit increased stability, increased resistance to proteolytic inactivation, decreased / non-existent immunogenicity, increased

circulating lives and low toxicity. In addition PEG-modified proteins have been shown to be soluble in organic solvents yet retain activity (200).

The modification of proteins with PEG can confer a number of advantages and unique characteristics to the conjugate. From a clinical viewpoint the increase in circulatory life and a reduction in immunogenicity have had greatest impact. Smaller doses of PEG-enzyme should be needed, and the frequency of injections could be decreased facilitating patient management. In chemical terms, the enhanced solubility and stability of proteins conjugated to PEG has opened up attractive avenues of research.

The sharp rise in circulatory half life seen when proteins are modified with PEG is due to a change in a number of factors. However, it has been postulated that all these effects are due to the formation of a shell of PEG molecules around the protein. This is believed to sterically hinder recognition of the foreign protein by the immune system (201). In some cases modified proteins circulate similarly in virgin and immunised (to the protein) mice, indicating that the PEG adduct is non-immunogenic (194, 196, 202, 203), and therefore not cleared from the circulation by the immune system. The extent of immunogenicity, however, varies with each protein and the percentage of PEG attached to the protein surface (204). Steric hindrance may play an important role in preventing the uptake of protein conjugates by RME (receptor mediated endocytosis) (205), and the recognition by various circulating enzymes (195). It has also been shown (206) that proteins conjugated to PEG are more resistant to proteolysis and clearance through glomerular filtration by the kidney (205) than their unmodified counterparts, thus increasing their circulatory half life still further. Davis *et al* (207) reported little change in the immunogenicity or blood circulating lives of rIL-2 after repetitive injections. This important work revealed that maintaining systemic exposure caused no detrimental reaction by the immune system. This will be clinically important for patients under a long term medical regime.

As PEG is extremely hydrophilic, the formation of a water shell around the protein can explain the retention of biological activity of the protein in organic solvents and the increase in solubility (208). Solubility is increased to an extent whereby proteins which are normally insoluble under physiological conditions can be solubilised by PEG attachment (209). Solubility in organic solvents such as benzene, toluene, acetone, ethanol and dimethylformamide has also been demonstrated (210).

The activity of most proteins following modification by PEG is usually decreased (211), often as a result of conformational changes in the protein structure. However, this loss of activity is more than offset by the concomitant increase *in vivo* in circulating life in the blood stream [*see table 1.1*]. Several cases of enhanced activity obtained as a result of PEG attachment to proteins have been described in the literature. Beauchamp (212) and Zalipsky *et al* (213) both observed enhanced activity of PEG-modified trypsins toward substrates. Naoi *et al* reported an enhanced activity of PEG-β-galactosidase toward hydrophobic substrates and substantial activity toward GM_1-ganglioside (214).

To summarise, a clinically useful enzyme appropriately modified with PEG would have:-

(1) an increased circulatory life.

(2) a reduction in immunogenicity and antigenicity.

(3) minimal loss of biological activity.

Table 1.1. A comparison of the half life of various PEG modified proteins.

Enzyme	Source	Route	Host	$t_{1/2}$ native	$t_{1/2}$ mPEG	Ref.
Adenosine deaminase	calf intestine	IV	Balb/c mice	30 min	28 min	326
Asparaginase	*E.coli*	IP	rats	2.9 h	56 h	327
Arginase	Bovine liver	IV	B6D2 F1/J mice	1 h	12 h	328
Catalase	Bovine liver	IV	Long-Evans mice	10 min	> 4 h	329
Superoxide dismutase	Bovine RBC	IV	Lewis-Male mice	5 min	4.20 h	330
Uricase	*Candida utilis*	IV	human	< 3 h	8 h	331

1.14. The Project.

It is clear from the work cited in this preliminary introduction that both PEG monomers and biodegradable oligopeptide sequences offer interesting opportunities for further development. With the exception of Ulbrich's paper on PEG containing enzymatically degradable bonds (341), research into biodegradable copolymers with oligopeptide chains has largely been over-shadowed by HPMA work, (*Section 1.8*).

Ulbrich successfully demonstrated the degradation of an oligopeptide sequence attached pendantly to a PEG polymer backbone, with the release of a fluorescent dye. It is proposed that by incorporating an oligopeptide in series with a suitably derivatised PEG monomer the degradation rate of the peptide chain will remain unchanged. It is expected therefore that a copolymer comprised of alternating PEG and oligopeptide will be susceptible to enzymatic degradation. Such a copolymer could be suitable for *in vivo* applications since complete degradation of the polymer would produce discrete PEG monomers capable of traversing the cell membrane and ultimately being excreted. In addition by carefully selecting the size of PEG monomer the solubility of the peptide / PEG conjugate could be enhanced providing a biodegradable, soluble copolymer.

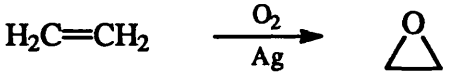
Chapter 2. The Epoxides.

2.1. Strategy.

The reaction of α,ω-bis(methylamino)PEGs with α,ω-bis(glycidyl)peptides was chosen as the copolymerisation strategy . The ability to generate the α,ω-bis(glycidyl)-peptides was studied using three approaches. In the first a bis-allyl was treated with the peroxy acid, MCPBA to affect the conversion of the olefin to an epoxide. In an alternative strategy, the peptides were synthesised between (methoxy)aryl or (benzyloxy)aryl groups. Hydrogenation of the less robust α,ω-bis(benzyloxy)peptides affected debenzylation and the phenolic hydroxy groups were subsequently converted to the α,ω-bis(glycidylethers) by treating with epichlorohydrin.

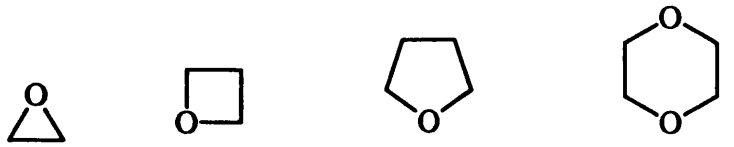
2.2. Introduction. The Epoxides.

Heterocyclic compounds are cyclic structures in which one or more ring atoms are hetero atoms, a hetero atom being an element other than carbon. The three membered rings, containing oxygen, are readily prepared from alkenes and because of this they are sometimes referred to as olefin oxides. The simplest heterocycle is ethylene oxide which can be called an epoxide, but is formally recognised by IUPAC as an oxirane. Ethylene oxide is a significant commercial item and is prepared industrially by the catalysed air oxidation of ethylene, (*Scheme 2.1*).



Scheme 2.1

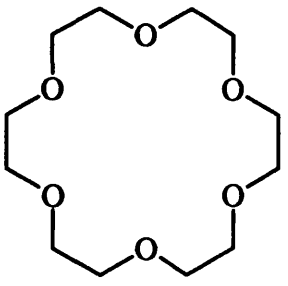
Due to the ring strain associated with the three membered ring, epoxides are much more reactive than other ethers. However, the epoxides are cleaved quite easily and protonated epoxides more readily still. The oxetanes are four-membered ring ethers which are more stable than the epoxides to ring opening reactions, but are still cleaved more readily than open chain ethers, (*Figure 2.1*).



Oxirane Oxetane Tetrahydrofuran 1,4-Dioxane

Fig. 2.1.

One of the most important of the cyclic ethers is tetrahydrofuran (THF), which is frequently used as a solvent. The five-membered ring is generally stable to ring opening, but this can be accomplished under conditions that cause the ether cleavage reactions of open chain ethers. A final group of large ring polyethers that have attracted a good deal of attention is the crown ethers. These compounds are cyclic polymers of ethylene glycol, $(\text{-}OCH_2CH_2)_n$ and are important for their ability to solvate cations strongly, (*Figure 2.2*).

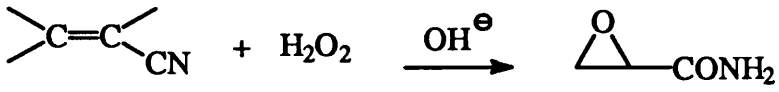


18-Crown-6 *Fig. 2.2.*

2.3. Methods of Epoxidation (addition of oxygen).

2.3.1. Hydrogen Peroxide.

It has been reported by Payne that H_2O_2 has been successfully used for the epoxidation of acrolein and acrylonitrile under conditions of controlled pH (215), (*Scheme 2.2*).

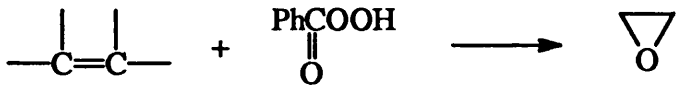


Scheme 2.2.

H_2O_2 is, by itself a relatively poor oxidising agent and to achieve epoxidation it must be 'activated' by conversion to another species. In the above reaction the intermediate is probably a peroxycarboximidic acid (216). Another remarkably powerful intermediate is trifluoroperoxyacetic acid which is prepared by the reaction of trifluoroacetic anhydride with 85 - 92% hydrogen peroxide (so called *high test* H_2O_2). The use of this reagent is however severely limited by the lack of stability.

2.3.2. The peroxidic reagents.

Olefins can be epoxidised with any of a number of peracids of which perbenzoic acid is the most commonly used, (*Scheme 2.3*). The reaction, which is called the Prilezhaev reaction has wide utility. Alkyl, aryl, hydroxyl, ester and other groups may be present, though not amino groups, since these are not affected by the reagent. Electron-donating groups increase the rate, and the reaction is particularly rapid with tetraalkyl olefins. Conditions are mild and oxiranyl yields are high.

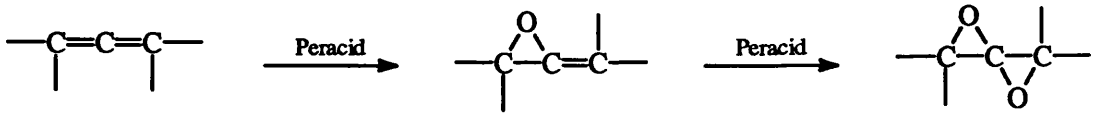


Scheme 2.3.

The peroxycarboxylic acids are generally unstable and must be stored in the cold, or preferably be prepared as needed. An important exception to this is 3-chloroperoxy benzoic acid (MCPBA) which is a stable crystalline solid. Other peracids, especially peracetic acid and trifluoroperacetic acid (217) are also reported. Conjugated dienes can be epoxidised although the reaction is slower than for the corresponding olefins.

α,β-Unsaturated ketones do not generally give epoxides when treated with peracids, for exceptions refer to Hart (218). However, MacPeek reported that α,β unsaturated esters react normally to give glycidic esters (219). When a carbonyl is in the molecule but not conjugated with the double bond, the Baeyer-Villiger reaction may compete.

Allenes are converted by peracids to allene oxides [1] or spiro dioxides [2] which are often unstable and react further to give other products, (*Scheme 2.4*).



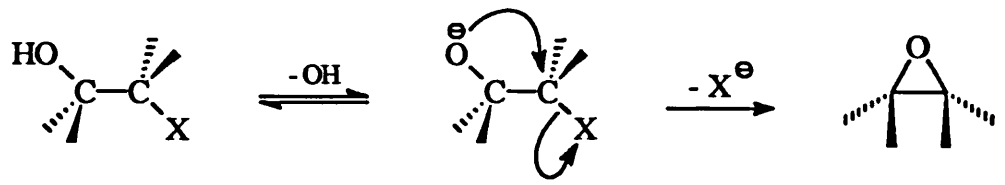
(1). (2).

Scheme 2.4.

Peracids react with C=N to give oxaziridines.

2.3.3. Cyclisation of halohydrins.

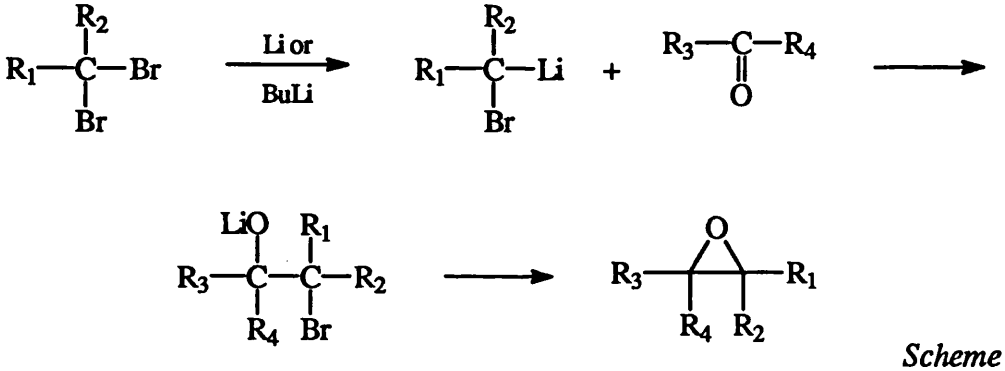
Many epoxides can be prepared through a displacement reaction involving the dehydration of a β-halo alcohol, (*Scheme 2.5*).



Scheme 2.5

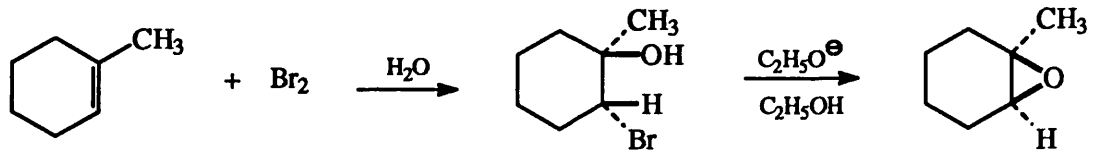
The base removes the proton from the OH group and the epoxide then attacks in an internal S_N2 aliphatic nucleophilic substitution reaction. The method can also be used to prepare larger cyclic ethers; five and six-membered rings.

Cainelli *et al* suggested that the *gem* dihalides treated with a carbonyl compound and lithium or butyl lithium gave epoxides (220), (*Scheme 2.6*).



Scheme 2.6.

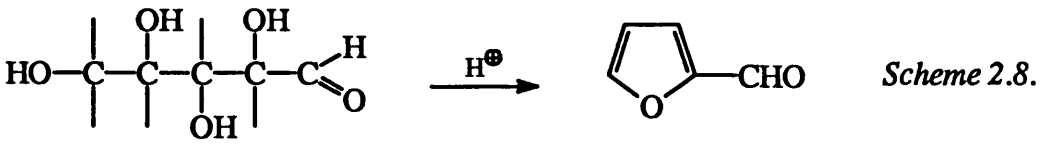
An alternative effective oxidation of the double bond may be achieved by the two-step addition of aqueous halogen followed by base-catalysed cyclisation, (*Scheme 2.7*).



Scheme 2.7.

2.3.4. Dehydration of alcohols.

Alcohols which have a hydrogen in the δ position can be cyclised with lead tetraacetate (221), (*Scheme 2.8*). The reaction has also been carried out with a mixture of halogen (Br_2 or I_2) and a salt or oxide of silver or mercury (especially HgO or AgOAc) (323).



Scheme 2.8.

Glycols can be converted to cyclic ethers by dehydration, although the reaction is most successful for five-membered rings, thus tetrahydrofurans can be formed in high yields.

2.3.5. Carbon adding to carbon.

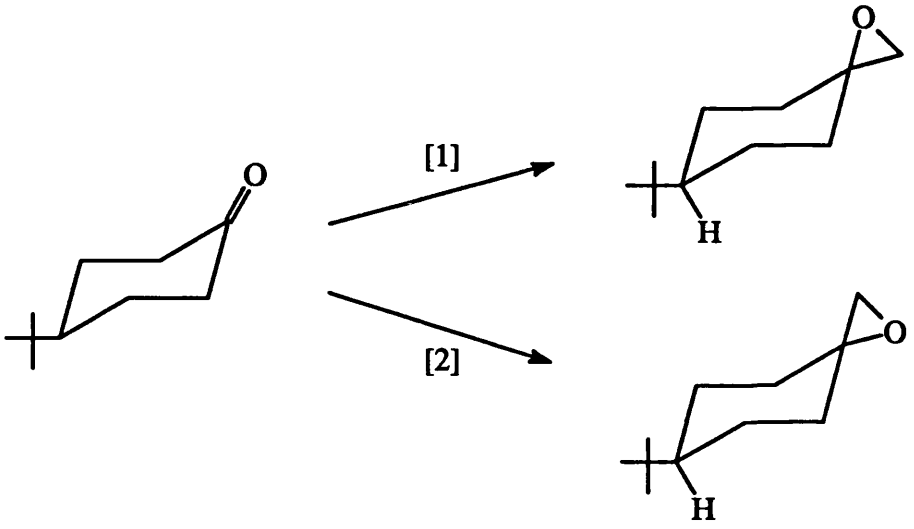
Aldehydes and ketones can be converted to epoxides in good yields with sulphur ylides (222) such as dimethyloxosulphonium methylide [1], (*Scheme 2.9*);

Scheme 2.9.

and the dimethylsulphonium methylide [2], (*Scheme 2.10*);

Scheme 2.10.

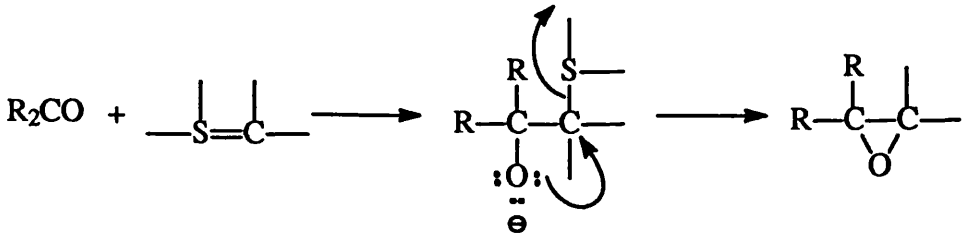
When diastereomeric epoxides can be formed, these two reagents attack at different sites. Thus 4-*t*-butylcyclohexanone treated with [1] and [2] will give the products shown below, (*Scheme 2.11*). With reagent [1] the new bond is axial whereas with the latter the new bond is equatoral.



Scheme 2.11.

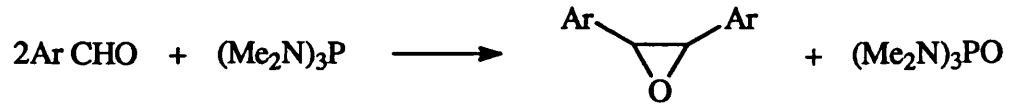
Another difference in behaviour between [1] and [2] is that with α,β unsaturated ketones, [1] gives only cyclopropanes, while [2] leads to formation of the oxirane.

Other sulphur ylides have been used in an analogous manner to transfer CHR or CHR_2, *e.g.* $Me_2S{=}CHCOO^-$, $Me_2S{=}CHPh$, $Me_2S{=}CH$-vinyl. The generally accepted mechanism is described, (*Scheme 2.11(a)*).



Scheme 2.11(a)..

Aromatic aldehydes can be dimerised to epoxides by treatment with hexamethyl phosphorous triamide, (223) (*Scheme 2.12*).

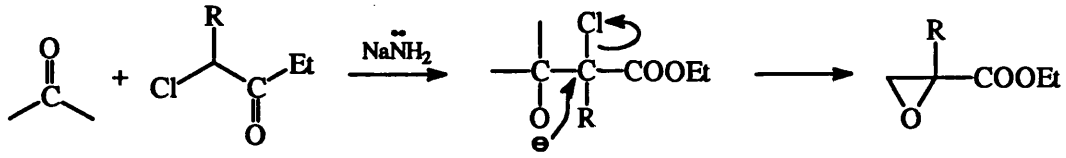


Scheme 2.12.

The reagent is converted to hexamethylphosphoric triamide (HMPT). The reaction can be used for the preparation of mixed epoxides by the use of a mixture of two aldehydes in which the less reactive aldehyde predominates.

2.3.6. Darzens glycidic ether condensation.

Aldehydes and ketones condense with α halo esters in the prescence of bases to give α, β epoxy esters called glycidic esters in good yields, (*Scheme 2.13*). For a more complete review the reader is directed to Ballester (224).



Scheme 2.13.

The reaction consists of an initial condensation followed by an internal S_N2 reaction. The Darzen reaction has also been carried out on α-halonitriles, α-halo sulphones, α-halo N,N-disubstituted amides and even on benzylic halides.

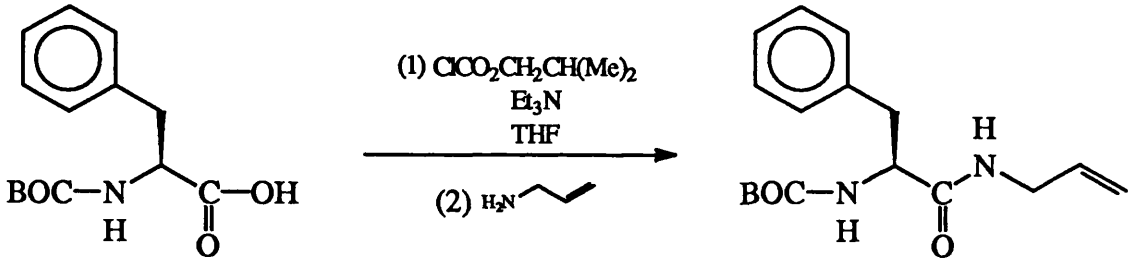
2.4. Discussion

To provide the appropriately derivatised peptides for polymerisation, it has been necessary to investigate a number of experimental strategies. The target polymers are formed by the copolymerisation of nucleophilic PEG monomers, and short peptide sequences derivatised with complimentary electrophilic groups. The glycidylether-(peptide) derivatives were chosen as a suitable electrophile for this purpose as they show high reactivity and are easily prepared in good yield.

As a model for the peptide chain, L-phenylalanine was chosen for investigation into the feasibility of forming a glycidyl ether directly onto the peptide chain. Phenylalanine is a convenient, commercially available model to use, as it possesses a chromophore which can be visualised easily on T.L.C. using U.V. light. This allows the experimental progress of a reaction to be monitored. This amino acid is also an integral part of the final tetrapeptide sequence and as such will reflect the chemical strategy eventually adopted.

Peroxybenzoic acid was discussed at length in Section 2.3.2. to generate oxiranes and has been found to be an efficient reagent for epoxidation of alkenes. By generating an unconjugated terminal alkene on both N and C terminals of phenylalanine and treating with peroxybenzoic acid, the bis(oxiranyl) amino acid can be generated.

To this end, the N-protected amino acid, BOCphenylalanine (*Compound 1*) was treated with isobutyl chloroformate and triethylamine. The resulting electrophilic mixed anhydride coupled with allylamine to give the N-allylamide, (*Scheme 2.14*).



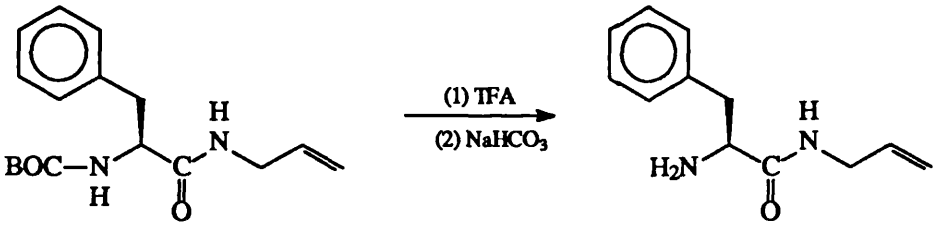
Compound 1. *Compound 2.*

Scheme 2.14.

The first of the attempts to make *Compound 2* involved adding one equivalent of allylamine quickly to the anhydride, at 0°C. Analysis by NMR revealed a mixture of products comprising predominantly of the isobutyl esters. In order to push the reaction towards the desired product, an excess of allylamine was used in later preparations, and was added dropwise at - 40°C. Conventional work-up afforded a crude product which

was recrystallised from light petroleum to give the amide in good yield. This material was identified as the allylamide by proton NMR spectroscopy. Peaks in the aliphatic region, δ 3.69 - 5.74, of the spectra were indicative of the alkene and were corroborated by mass spectroscopy, (*m/z* 305 (M + H)) and elemental analysis.

The BOC protecting group was removed by treating the BOCPhe-N-allylamide directly with an excess of trifluoroacetic acid (TFA) (225). The mixture was neutralised with aqueous potassium carbonate to give the free base phenylalanine-N-allylamide in good yield (*Compound 3*) (*Scheme 2.15*). Similar deprotection was also achieved using hydrogen chloride in dichloromethane. Deprotection of *Compound 2* was successful in both cases according to ^{1}H NMR which showed the disappeareance of the *t*-butyl peak at δ 1.3. A method involving cationic Dowex ion-exchange resin refluxed with the protected amino acid was attempted with no success.



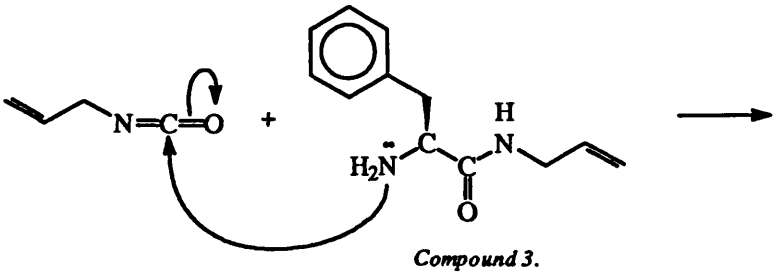
Scheme 2.15.

Compound 3.

To attach the second alkene an excess (2 eq.) of allyl isocyanate was added to phenylalanine-N-allylamide, (*Scheme 2.16*) with the formation of the urea. *Compound 4* was obtained in good yield, and was characterised fully by ^{1}H NMR. Mass spectroscopy revealed a parent ion peak at *m/z* 288.

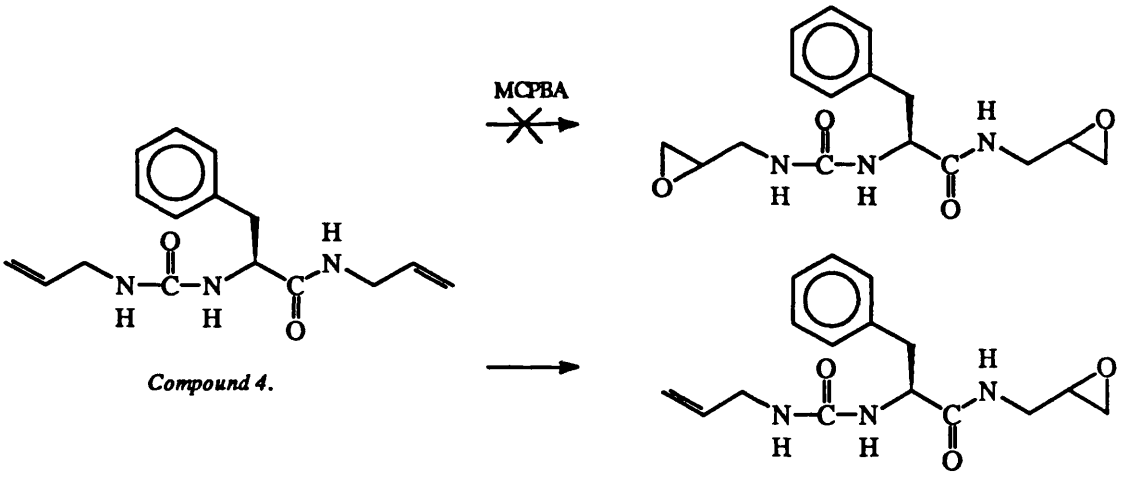
The terminal double bonds that were present in *Compound 4* should be, according to the literature (226), susceptible to epoxidation using the peroxybenzoic acids. MCPBA was chosen as a suitable reagent as it provides a simple one step route to epoxide formation. However, a mixture of *Compound 4* and MCPBA (3 eq.) which was boiled in dichloromethane failed to give any material that could be identified as the bis(oxiranyl)

compound. Further analysis of the isolated product by ^1H NMR revealed a confusing spectrum, containing multiple peaks within the aliphatic region. This region of the spectrum corresponds to the approximate shift values of the oxiranyl groups. Although full structural assignment was not possible it appeared that this material contained one oxiranylmethyl group and one allyl group. A series of model experiments were therfore undertaken to predict which of the allyl groups had been epoxidised and which was resistant to MCPBA.



Compound 3.

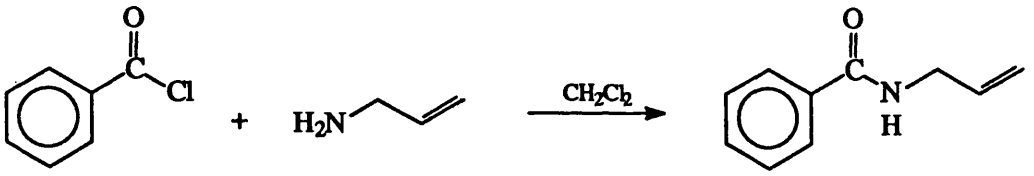
Scheme 2.16.



Compound 4.

2.4.1. Modes for the epoxidation reaction

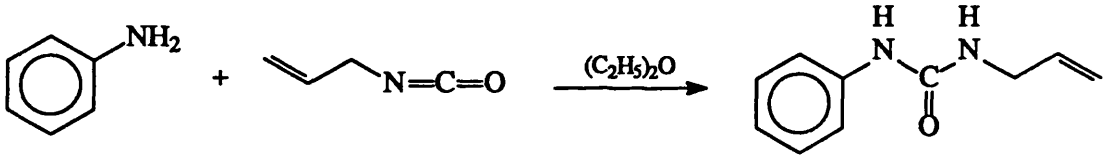
To investigate which olefin was susceptible to epoxidation with MCPBA, analogues bearing terminal allyl groups were synthesised. The first analogue was synthesised by treating benzoyl chloride with an excess of allylamine to form N-(allyl)benzamide, *Compound 5*, (*Scheme 2.17*). The amide was formed in good yield after a conventional work up. The broad singlet in the ^1H NMR at δ 6.97 could only be assigned to the newly formed amide NH.



Compound 5.

Scheme 2.17.

For the second analogue, *Compound 6*, reaction of aniline with allyl isocyanate gave the urea in moderate yield after careful trituration with heptane, (*Scheme 2.18*).



Aniline

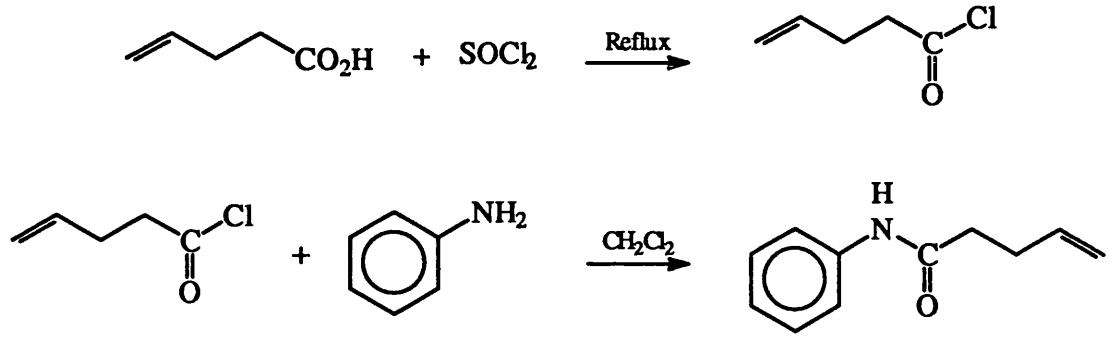
Compound 6.

Scheme 2.18.

Characterisation with ^{1}H NMR revealed the loss of the aniline primary amine peak circa δ 9.5 ppm and the formation of the two amides NHs at δ 7.62. Further data from mass spectroscopy showed a parent ion at *m/z 176*. The formation of the urea in low yield was uncharacteristic of this type of reaction, where the reaction of the isocyanate involves nucleophilic attack on the multiply-bonded carbon, followed by the addition of a proton to nitrogen to form the urea. It was noted that the aniline used for the experiment showed signs of deterioration which may have introduced impurities into the reaction.

It was hypothesised that the third analogue N-phenylpent-4-enamide (*Compound 7*) (*Scheme 2.19*) may be more susceptible to epoxidation. By introducing pent-4-enoic acid, an extra CH_2 is incorporated between the olefin and the aromatic ring reducing any aryl influence.

The acid chloride of pent-4-enoic acid was isolated by distillation and added to an excess of aniline to give the known amide *Compound 7* in good yield, (227).

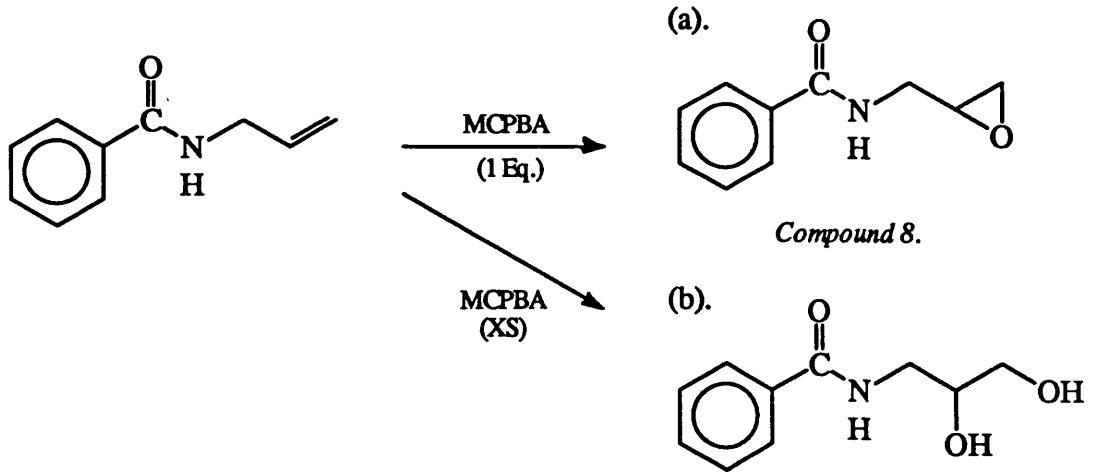


Compound 7.

Scheme 2.19.

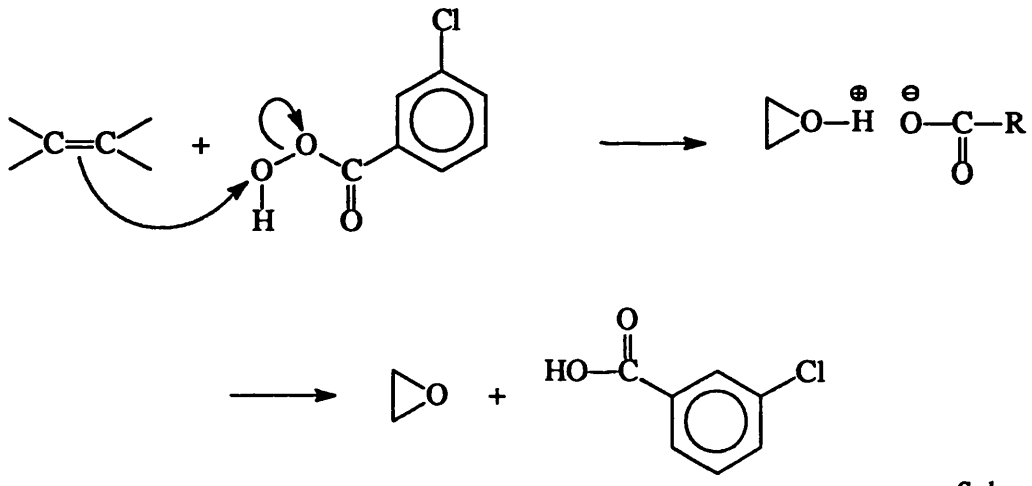
The primary route to the formation of an oxirane with N-(allyl)benzamide was to boil the olefin in dichloromethane with an excess of MCPBA (3 eq.). Chromatography gave the expected oxirane (*Compound 8*) and the corresponding diol (*Scheme 2.20*), as revealed by mass spectroscopy (M + H at *m/z* 177 and M at *m/z* 195, respectively).

Consequently the experimental protocol was modified to reduce the amount of MCPBA (1 eq.) and avoid washing the product with weak acid. Following column chromatography, a small amount of the product was isolated as an oil but the majority of the material was recovered as the N-(allyl)benzamide starting material, suggesting that the MCPBA was not the cause of the ring opening. The mechanism can be represented as a displacement reaction on an electrophilic oxygen by a nucleophilic alkene, (*Scheme 2.21*).



Scheme 2.20.

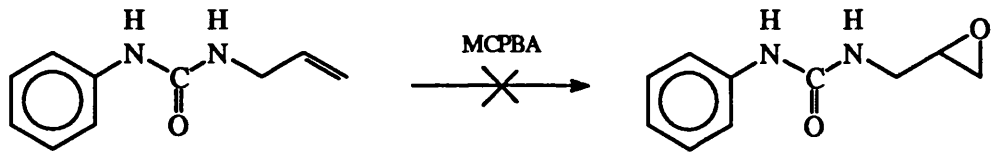
The mechanism of epoxidation by MCPBA.



Scheme 2.21

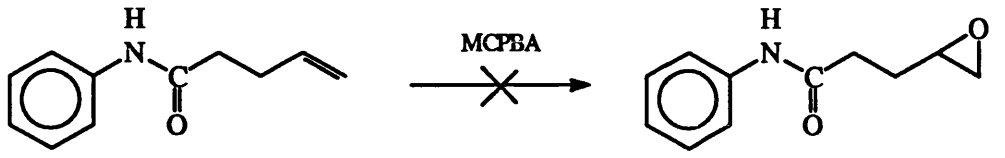
Treatment of the urea of *Compound 6* and the amide of *Compound 7* with MCPBA in refluxing dichloromethane gave no material which could be identified as products of oxidation, (*Scheme 2.22*).

The results with model compounds suggest that the bis-allyl phenylalanine derivative (*Compound 4*) had been epoxidised solely at the N-allyl amide and not at the N-allyl urea. The mechanism of this selectivity is obscure and was not investigated.



Compound 6.

Scheme 2.22.



Compound 7.

2.4.2 Using phenols as points of attachment of epoxides.

The unforeseen problems thrown up by attempting to generate an oxirane on a peptide group encouraged new approaches to be investigated. One attractive strategy was to completely avoid synthesising the oxirane and instead incorporate a compound already containing the group epichlorohydrin, (*Figure 2.3*).

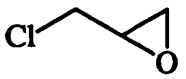


Fig. 2.3.

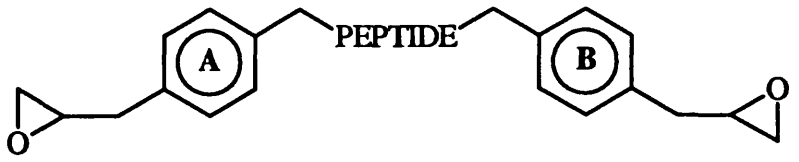
A linker was therefore required that was capable of carrying an epoxide but was also sympathetic to coupling at both N- and C- peptide terminals, (*Figure 2.4*).

Fig. 2.4.

To provide an appropriately derivatised linker, it was necessary to synthesise various protected phenols. Various groups were investigated for their ability to : (a) couple to the peptides; (b) carry a suitable epoxide-containing compound.

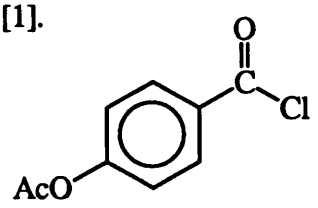
Figure 15 lists a series of orthogonal structures with various side protecting groups for the left (A) and right (B) side of the target compound. All the compounds listed in series (A) offer the opportunity to attach amino acids *via* the peptidic nucleophilic primary amine and a suitable coupling reagent. On the contrary, series (B) includes aryl amines, with functional groups susceptible only to electrophilic substitution at the peptidic carbonyl group. All the compounds listed within the table possess *para* phenols or protected phenols for reaction with epichlorohydrin. It is not within the scope of this chapter to discuss the incorporation of peptide sequences between the phenyl groups, and the reader is directed to Section 3.5.

Experimental Strategy. ***Fig. 2.5.***

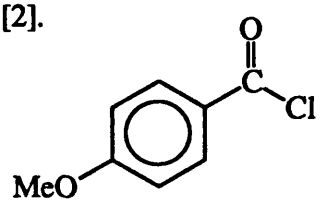


Series A.

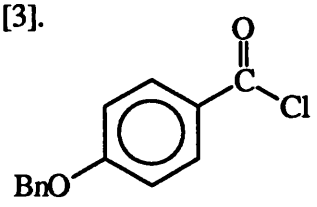
[1].



[2].

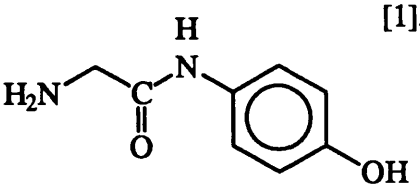


[3].

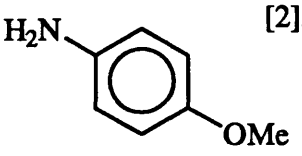


Series B.

[1].



[2].



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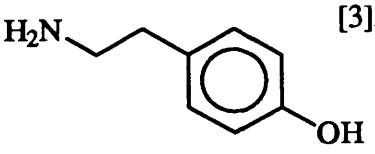
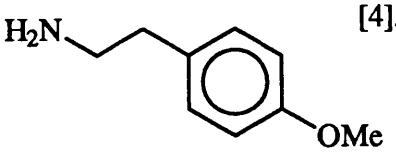
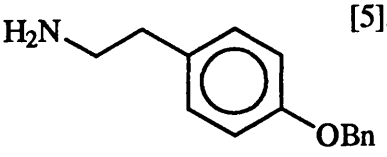


[4].

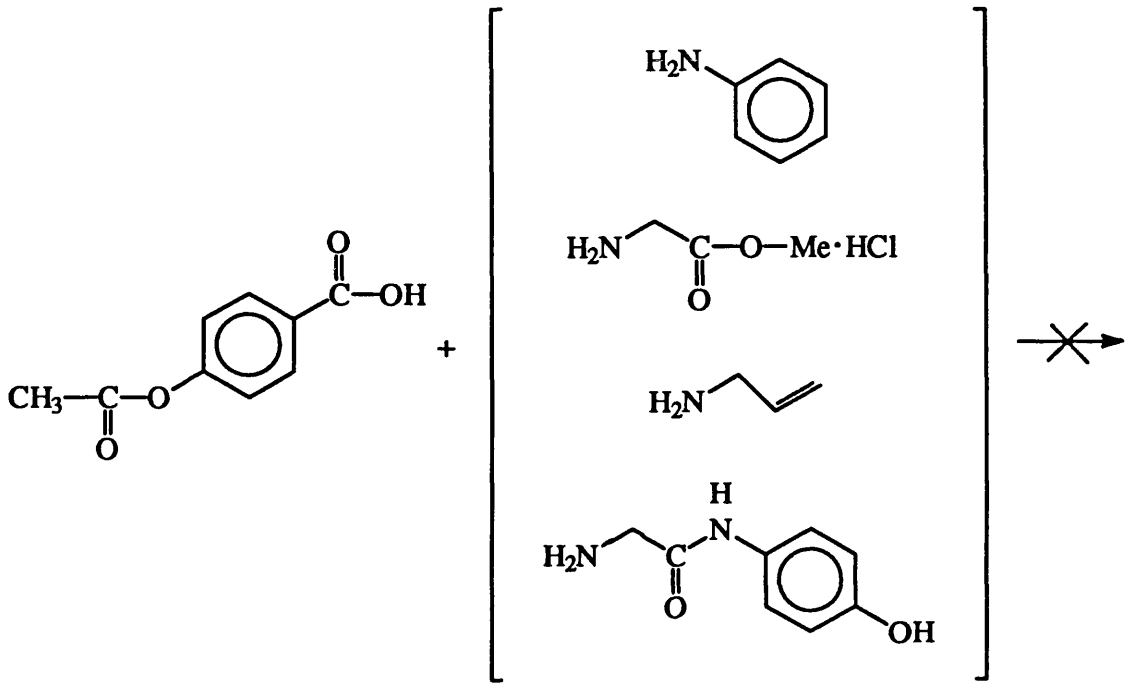


[5].



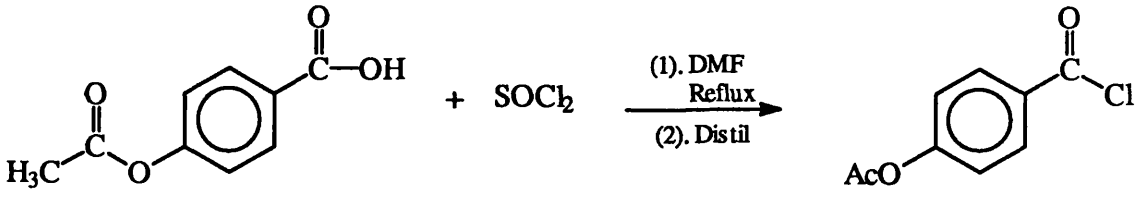
Preliminary work was initiated using 4-acetoxybenzoic acid, A[1] in which the phenol is protected as an ester. Repeated attempts were made to couple the acid to

compounds containing primary amines in an attempt to show that the attachment of peptides was possible. However, neither aniline, allylamine, N-2-(4-hydroxyphenyl) glycinamide or glycine methyl ester hydrochloride were successfully coupled despite attempts using a variety of coupling reagents, (*Scheme 2.23*).



Scheme 2.23.

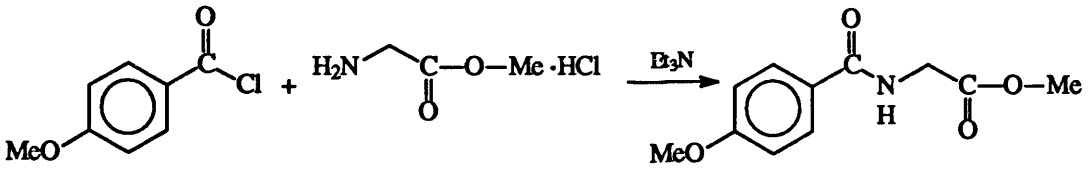
The reason for the lack of reactivity of A[1] was unclear. In a reaction designed to increase the reactivity of 4-acetoxybenzoic acid to nucleophiles the acid was converted to the acid chloride (*Compound 9*), (*Scheme 2.24*), in a reaction catalysed by dimethylformamide. Reaction of this acid chloride with amines was however fraught with technical difficulties.



Compound 9.

Scheme 2.24.

In order to test the utility of a more robust protecting group for the phenol, 4-methoxybenzoyl chloride was investigated. Compound A[2] reacted successfully (*Compound 10*) with a primary amine (glycine methyl ester hydrochloride) (*Scheme 2.25*) in the presence of triethylamine.



Compound 10.

Scheme 2.25.

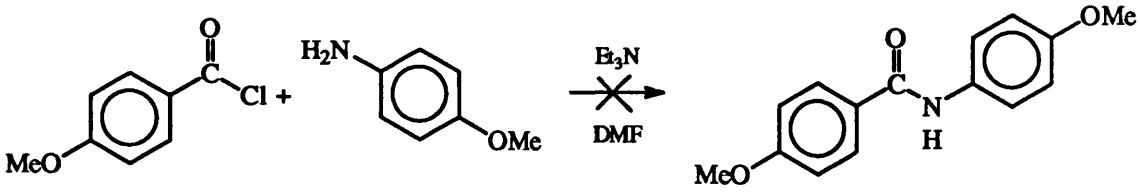
The ability to initiate a peptidic sequence on one of the methoxybenzoyl compounds encouraged the epoxidation process to be studied in more detail.

2.4.2.1. The 4-methoxybenzoyl peptide work.

To generate a series of models for further investigation 4-methoxybenzoyl chloride was coupled to a number of compounds from the Series (B). The overall strategy was to attempt to convert the *para*-side group protecting derivatives into functionalities bearing an epoxide, before any amino acids were introduced into the compound.

2.4.2.1.(a). 4-Methoxyaniline.

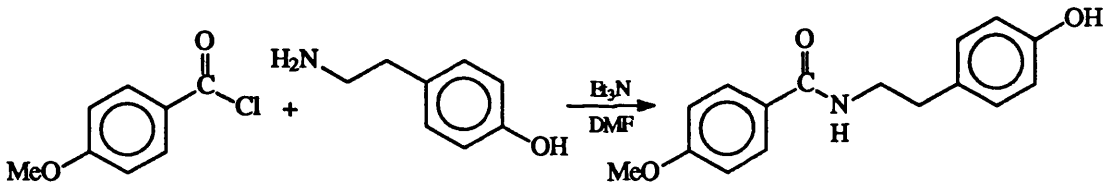
4-Methoxyaniline, (Anisole B[2]) showed no reactivity at all with 4-methoxy-benzoyl chloride A[2] (*Scheme 2.26*) or with the activated esters of N-(4-(methoxy-benzoyl)glycine.



Scheme 2.26.

2.4.2.1.(b). 2-(4-Hydroxyphenyl)ethylamine.

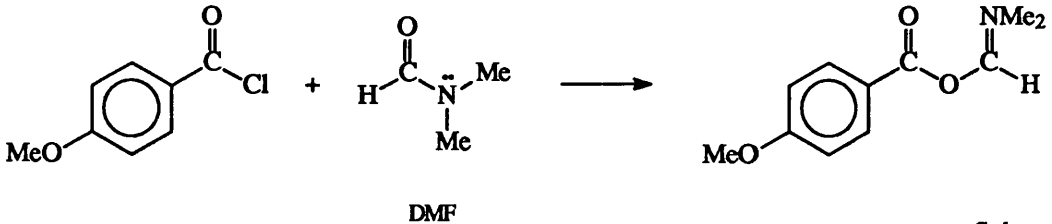
Tyramine (2-(4-hydroxyphenyl)ethylamine) B[3] was chosen as a candidate for further work because of the extended alkyl chain bearing an amino group. By increasing the distance of the amino group from the ring system, the nucleophilic nature of the amine is increased and problems due to steric hindrance and delocalisation from the phenyl are decreased. 2-(4-Hydroxyphenyl)ethylamine was successfully allowed to react with 4-methoxybenzoyl chloride in good yield according to *Scheme 2.27* to form *Compound 11.*



Compound 11.

Scheme 2.27.

A solution of triethylamine and tyramine were prepared in dimethylformamide prior to carefully adding 4-methoxybenzoyl chloride. If the 4-methoxybenzoyl chloride is first dissolved in DMF there is a danger that the dimethylformamide will initiate a reaction with the chloride before the 2-(4-hydroxyphenyl)ethylamine is introduced, (see *Scheme 2.28*).



DMF

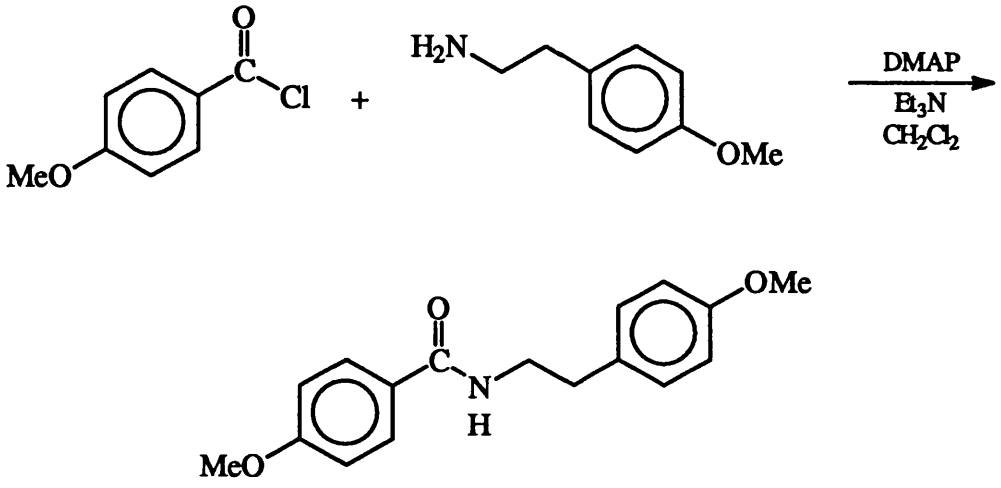
Scheme 2.28.

The oxygen which is present on dimethylformamide has a greater electronegativity than the amino group present on 2-(4-hydroxyphenyl)ethylamine. If dimethylformamide and anisoyl chloride are mixed then the oxygen will be allowed to react in preference to the amine and no product will be formed. *Compound 11* was extracted from the reaction medium by the addition of water and collected by filtration in good yield. The material was identified as the monomethoxy, monohydroxy product by ^{1}H NMR with the singlet peaks of the methoxy group at δ 3.78 and hydroxy group at δ 9.15 giving equivalent interaction with the amide NH multiplet at at δ 8.42. This characterisation was also strengthened by the observation of the M + H ion at *m/z* 271 in the mass spectrum.

2.4.2.1.(c). 2-(4-Methoxyphenyl)ethylamine.

At the same time, additional work was successfully progressing with a compound analogous to tyramine. 2-(4-Methoxyphenyl) ethylamine, B[4], also contains an alkyl amino group at a distance from the aromatic ring, but has the phenyl protected as the methyl ether (*Scheme 2.29*). The additional methoxy protecting group introduced by this reaction was thought to be advantageous in a number of ways:- *1.* the overall solubility of the compound in organic solvents was increased when compared to compound B[3] containing a hydroxyl; *2.* the phenolic OH group is comparitively reactive compared to the methoxy. By incorporating a methyl ether group to protect the phenolic side group any further reaction at this site, during peptide synthesis for example, is denied.

The acid chloride A[2] was treated with an amine B[4] in the presence of triethylamine (a tertiary amine) and DMAP (a nucleophilic catalyst). The synthesis of the bis(methoxy) compound was completed after a conventional work up afforded a crude product which was recrystallised to give *Compound 12* in good yield. The characterisation was substantiated by mass spectroscopy, M + H ion at *m/z* 286 and ^{1}H NMR characterisation where two singlets at δ 3.79 and δ 3.82 were assigned to the aryl methoxy groups.



Compound 12. *Scheme 2.29.*

2.4.3. The cleavage of the ethers

A great deal of research time was spent optimising a suitable experimental protocol to remove the methyl ether protecting groups from the aryl compounds prior to the inclusion of any peptides.

Ethers, particularly alkyl aryl ethers can be cleaved by heating with concentrated hydrogen bromide or hydrogen iodide (228). Hydrogen chloride is seldom successful. Hydrogen bromide reacts more slowly than the iodide, but it is often a superior reagent since it causes fewer side reactions. Ethers have also been cleaved with Lewis acids such as BF_3, BCl_3, BBr_3 (229, 230) or $AlCl_3$. In such situations, the departure of the alkyl group is assisted by complex formation with the Lewis acid, (*Figure 2.6*).

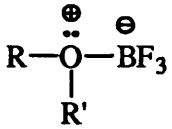
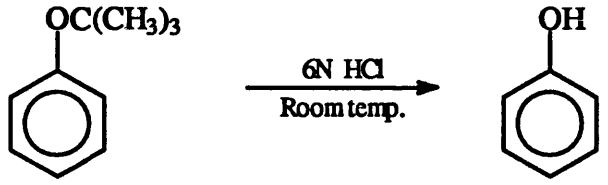


Fig. 2.6.

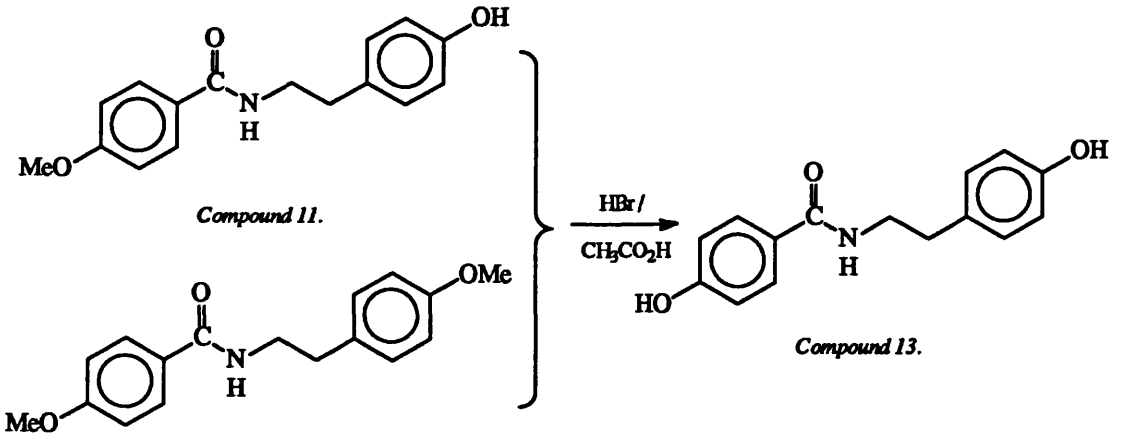
Alkyl aryl ethers can also be cleaved with lithium iodide to give alkyl iodides and salts of phenols (231). Dialkyl or aryl ethers may be cleaved with anhydrous sulphonic acids for

the aryl alkyl ethers, cleavage always takes place to give the phenol. Elkobaisi (232) reported that when a benzyl phenyl ether heated to 250°C for some days it undergoes thermal rearrangement to give *o*- and *p*-benzylphenols!. When R is a tertiary alkyl group, the ether cleavage is especially facile; cleavage occurs by the S_N1 mechanism (*Scheme 2.30*).



Scheme 2.30.

An attempt was made to adapt Burwell's synthesis of phenols by the nucleophilic substitution of the methoxy aryl protecting groups with hydrogen bromide, (*Scheme 2.31*). The bis(methoxy) and mono(methoxy) compounds (*Compounds [11] and [12]*) were boiled in an excess (10 eq.) of hydrogen bromide / acetic acid (30%). The reaction mechanism involves protonation of the ether which undergoes S_N1 or S_N2 cleavage. Because the phenyl group is not susceptible to either S_N1 or S_N2 reaction, cleavage of the aliphatic C-O bonds should always occur. The alkyl oxygen bond is broken and the phenolic compound is the leaving group (*Scheme 2.32*).



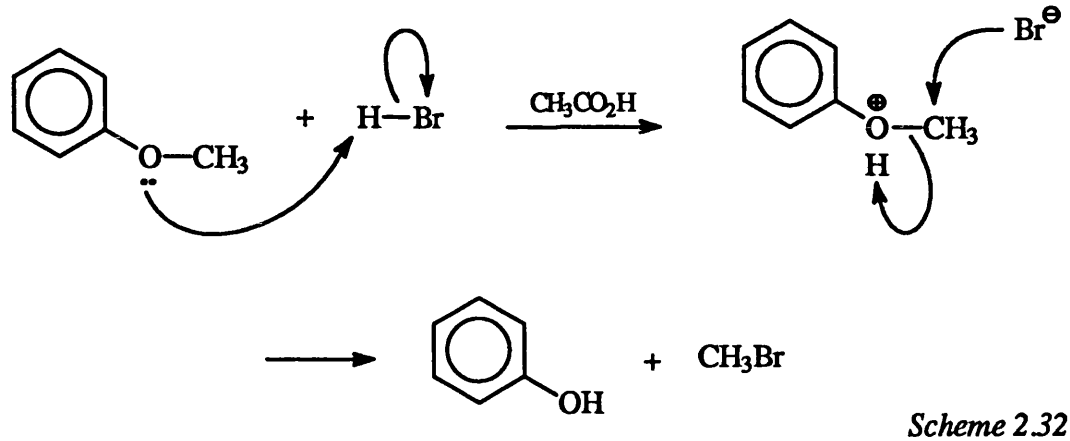
Compound 11.

Compound 12.

Compound 13.

Scheme 2.31.

Scheme 2.32. The stoichiometric mechanism for hydrogen bromide demethylation.

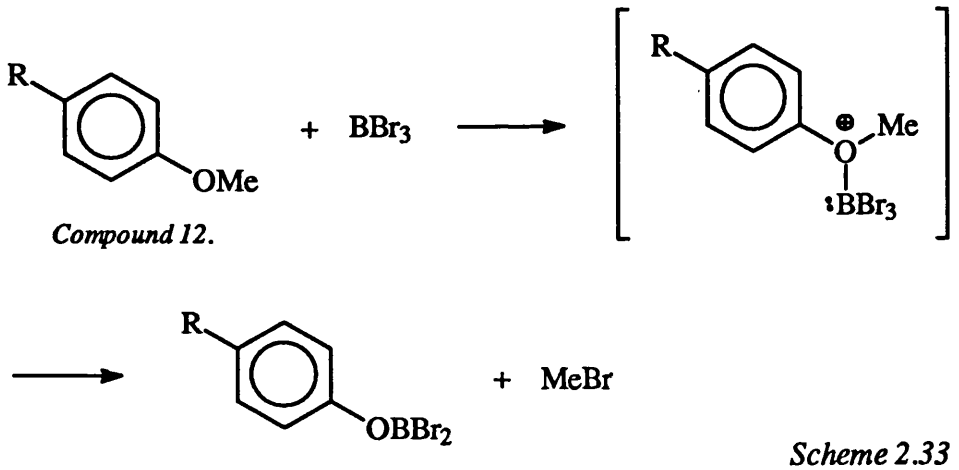


Scheme 2.32.

The results of the attempted ether cleavage using hydrogen bromide were not as successful as the literature reviews indicated. Typically the bis(methoxy) compound [*12*] was refluxed for 12 hours with an excess of hydrogen bromide (30%) in acetic acid. A crude product was isolated in poor yield that on further characterisation (^{1}H NMR) revealed a mixture of starting material and product. Subsequently, the reflux times were extended in addition to increasing the molar ration of hydrogen bromide to starting material. These changes appeared to have little effect on the cleavage of the aryl ethers, although a small amount of 4-hydroxy-N-(2-(4-hydroxyphenyl)ethyl)benzamide (*Compound 13*) was separated from the starting material by column chromotography. *Compound 13* was verified by ^{1}H NMR, which revealed that the two singlets corresponding to the methoxy groups had been replaced with two broad singlets at δ 9.0 and δ 10.0 corresponding to the formation of the diol compound.

Despite the poor yield of *Compound 13* the cleavage of aryl ethers was shown to be feasible. However, the conditions in which the alkyl groups were cleaved must be made more aggressive if there was to be an increased yield in the conversion of *Compound 12* to *Compound 13*. Boron tribromide has been used for effecting the complete demethylation of aryl methyl esters. McOmie *et al* (230) reported that aromatic ethers similar to

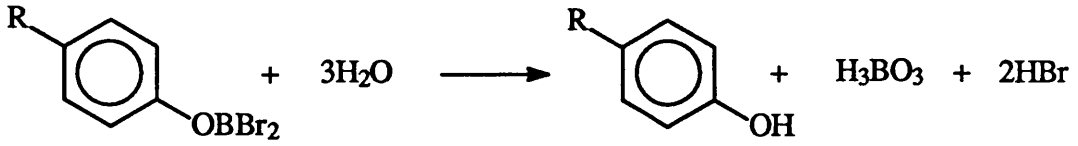
compound [*12*] were cleaved at or below room temperature by means of boron tribromide. Over twenty aryl ethers were reported to have been successfully cleaved, many of which were resistant to demethylation with hydrogen bromide or aluminium bromide. When the bis(methyl) compound [*12*] was allowed to react directly with boron tribromide in dichloromethane [1.0 M] and no other solvent, successful demethylation was achieved, (*Compound 14*). The reaction probably proceeded *via* a complex formed between the reagent and the ethereal oxygen atom, (*Scheme 2.33*). It was advisable therefore to use one mole of boron tribromide per ether group.



Compound 12.

Scheme 2.33

After the reaction was complete, the dibromide complex and excess reagent were hydrolysed by adding water to the reaction. The phenolic product quickly precipitated as a solid and was collected by filtration whilst the boric acid and hydrogen bromide remained in solution, (*Scheme 2.34*).



Compound 14.

Scheme 2.34.

Preliminary experiments with boron tribromide were largely disappointing as complete demethylation was not achieved. Simple characterisation (T.L.C., ^{1}H NMR) often revealed a mixture of starting material and product. This was alleviated in a number of ways. The molar ratio of boron tribromide was increased from 2 equivalents to 3.5 equivalents to take account of other potentially nucleophilic groups, *e.g.* the nucleophilic oxygen, which may have been allowed to react with the reagent thereby decreasing it's molarity, (*Figure 2.6*). In addition, the volume of solvent was increased and the reaction boiled in dichloromethane.

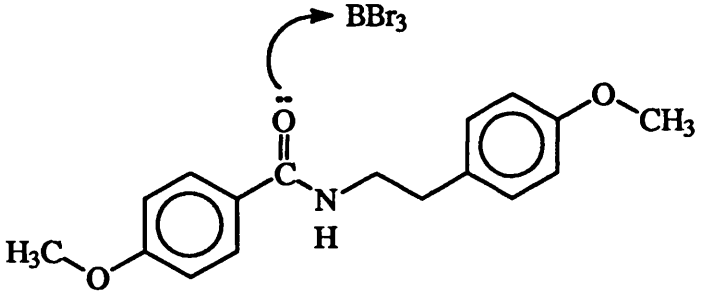


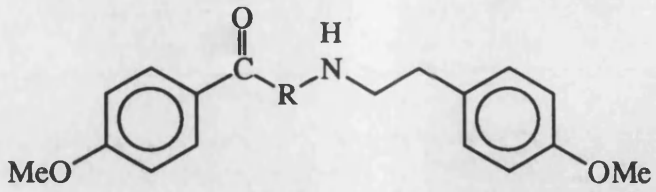
Fig. 2.6.

Compound 12.

Compound 14 (Scheme 2.34) was obtained in a good yield following recrystallisation from alcohol and ether. A narrow melting point was auspicious of a pure product which was verified through ^{1}H NMR. Elemental analysis was accurate and the parent ion peak at *m/z* 258 in the mass spectrum was further evidence of the correct product.

2.4.3.1. Peptide compounds and ether cleavage.

The synthesis of a number of di-, tri- and tetra- peptide sequences between orthogonal groups, protected by methyl ethers had been accomplished, (*Figure 2.7*). As a test substrate and target compound, the tetra peptide, GlyPheLeuGly (*Compound 15*) was proposed as a good candidate for further modification.

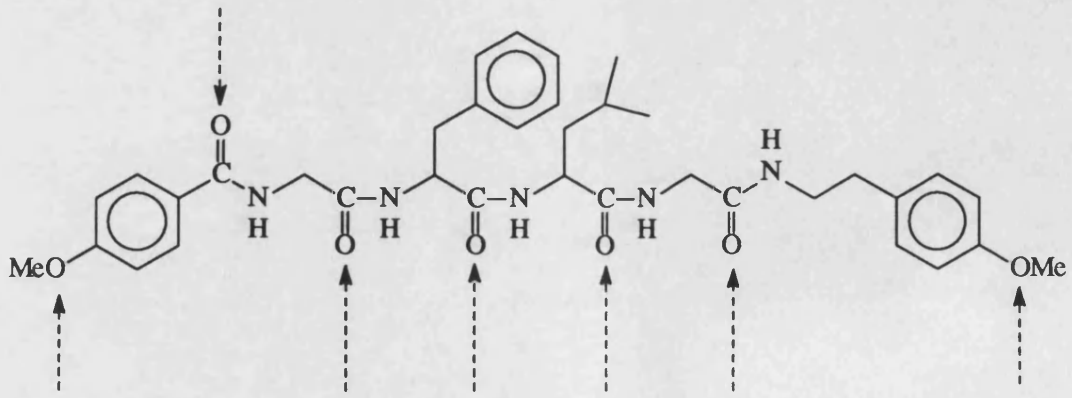


R = Gly, (Compound 27).
Gly Gly, (Compound 38).
Gly Phe Gly, (Compound 50).
Gly Phe Leu Gly, (Compound 15).

Fig. 2.7.

Using fresh boron tribromide / dichloromethane (1.0 M) (3.5 equivalents) the tetrapeptide was refluxed in dichloromethane for 12 hours. The reaction was frequently monitored by T.L.C. which indicated that the reaction was proceeding slowly, if at all. It was propounded that the incorporation of the tetrapeptide between the alkylaryl ethers was interfering in some way with the reaction of boron tribromide. The most likely scenario was that the nucleophilic oxygen on the peptidic carbonyl groups were complexing the electrophilic boron tribromide, (*Figure 2.8*). As a consequence, the molar ratio of boron tribromide was increased to 10 molar equivalents and the reaction was refluxed for a further 3 hours under nitrogen.

Diagramatic representation of the possible Lewis basic sites for BBr_3 interaction.



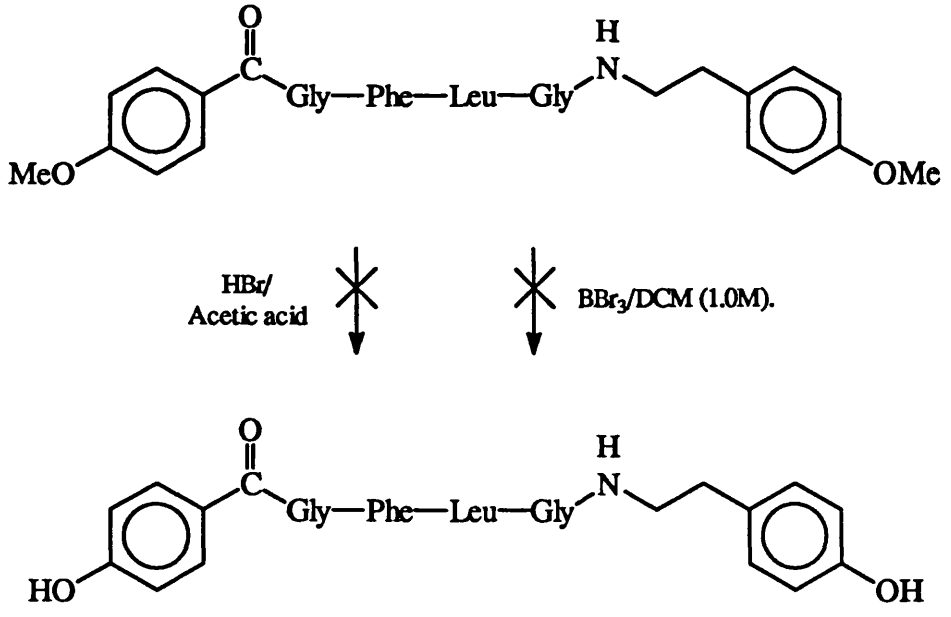
------► = Site of boron complexes.

Fig. 2.8.

Provisional T.L.C. using methanolic iron III chloride solution (to visualise phenolic OH groups) provided no evidence for the presence of the target phenolic compound. This was

corroborated with a more detailed characterisation. Using T.L.C. revealed a multi-component plate that could not be separated despite using an arsenal of solvent systems. The ^1H NMR spectrum was tremendously complicated but more importantly there was no evidence of any phenolic groups.

Despite the previous limited success with hydrogen bromide a further attempt was made using this reagent to demethylate the tetra-peptide compound. A huge excess of HBr / acetic acid (70 eq.), was boiled with the bis(methoxy) compound however, no material which could be identified as the required α,ω-bis(phenol) derivative was isolated from the reaction, (*Scheme 2.35*).



Scheme 2.35.

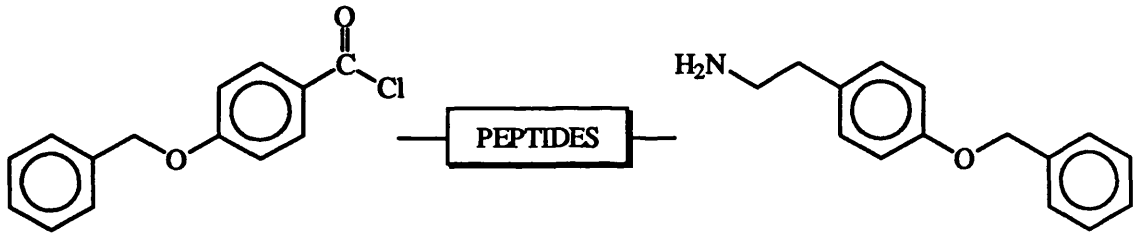
2.5. The use of aryl phenyl ethers.

Owing to the unforeseen problems associated with demethylation of products possessing bis (methyl) groups and a peptide sequence an alternative strategy was adopted. The revised putative precursors to epoxide derivatisation were synthesised according to the reaction schemes below. The phenolic functions were protected during

the syntheses as benzyl ethers. These could be easily removed, under milder conditions, by hydrogenolysis in a penultimate step before epoxide addition.

Unlike the methoxyaryl compounds which were commercially available the analogues shown in *Figure 2.9* were prepared from empirical compounds.

The Proposed Target Compounds.



4-(Phenylmethoxy)benzoyl chloride

Series A[3].

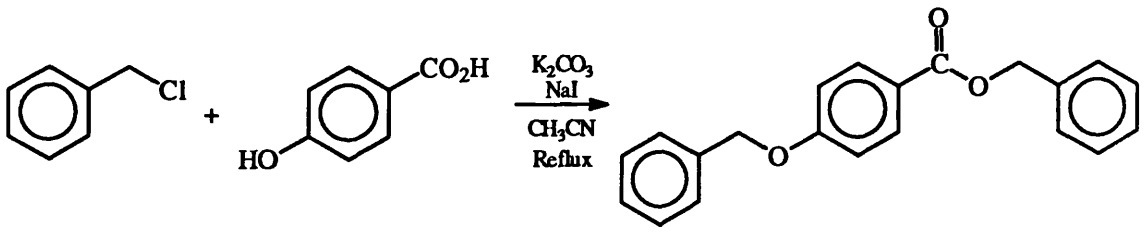
2-(4-(Phenylmethoxy)phenyl) ethylamine

Series B[5].

Fig. 2.9.

2.5.1. The Left Side. [4-(Phenylmethoxy)benzoyl chloride].

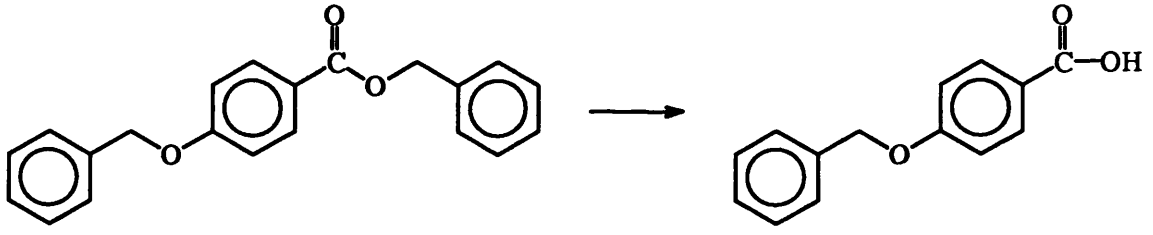
In a three step procedure, adapted from the method of Herbert *et al* (233), 4-hydroxy benzoic acid was initially refluxed in acetonitrile which contained benzyl chloride, potassium carbonate and sodium iodide, (*Scheme 2.36*).



Compound 16.

Scheme 2.36.

The product, phenylmethyl-4-(phenylmethoxy)benzoate had properties identical to those reported (233). *Compound 16* was converted to 4-(phenylmethoxy)benzoic acid (*Compound 17*) by hydrolysis which involved refluxing the benzoate with sodium hydroxide (2.0 M) and ethanol, (*Scheme 2.37*). The product was initially afforded as the sodium salt which would neither dissolve in water or organic solvent, at room temperature. However, once this intermediate was acidified with sulphuric acid (2.0 M), it could be extracted into warm ethyl acetate. Evaporation of the solvent afforded the product in good yield and characterisation with ^{1}H NMR established the loss of five protons in the integrated benzyloxy peak. The formation of a new broad peak at δ 12.6 was recognised as the hydroxyl functionality of the carboxylic acid

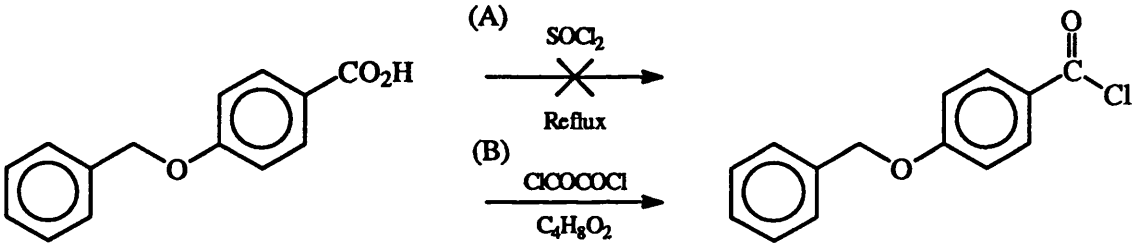


Compound 17.

Scheme 2.37.

In order to provide a suitably derivatised compound capable of reacting further with a peptide chain, the acid was converted into an acyl halide using thionyl chloride. This type of reaction is the best and most common method for the preparation of acyl chlorides. The use of thionyl chloride is attractive as the by products of the reaction are gases and the acyl halide is therefore easily isolated. 4-(Phenylmethoxy)benzoic acid (*Compound 17*) was boiled under reflux with thionyl chloride for 4.5 hours whereupon the thionyl chloride was removed by distillation, (*Scheme 2.38*). A black residue was produced which could not be dissolved or recrystallised in any of a number of solvents including ethyl acetate, dioxane, light petroleum or dichloromethane.

Oxalyl chloride and bromide are other, frequently used acyl halide reagents which on reaction form the acid chloride and decompose to carbon monoxide and carbon dioxide. A further attempt was made to make 4-(phenylmethoxy)benzoyl chloride (*Compound 18*) by using oxalyl chloride. The acid and oxalyl chloride (2 eq.) were dissolved in dioxane and one drop of DMF was added to catalyse the reaction. The reaction was complete when the evolution of gas had ceased whereupon the solvent was evaporated and the crude product was recrystallised from light petroleum.



Compound 18.

Scheme 2.38.

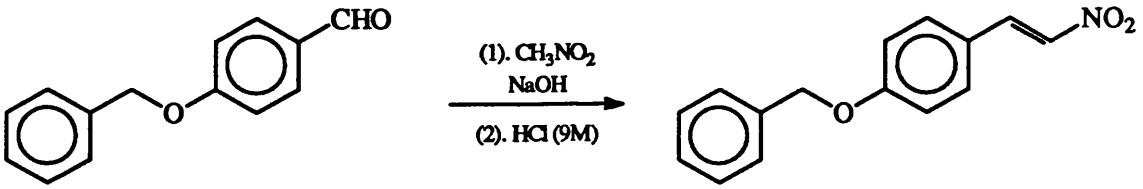
The acid chloride was characterised with I.R. which showed a migration of the carbonyl peak from 1600 cm^{-1} in the acid to 1775 cm^{-1} for the acid chloride carbonyl group. In addition the identity of the acid chloride was substantiated by ^1H NMR which detected the increased electron withdrawing effect of the acid chloride on the aryl protons ortho to the substituted group. The shift values for the doublet peak at position 2' - 6' in *Compound 17* were 7.89 with respect to 8.07 in *Compound 18* for the two protons.

2.5.2. The Right Side. [2-(4-(Phenylmethoxy)phenyl)ethylamine].

This method was adapted from Agorta (234) and involves condensation of nitromethane with 4-(phenylmethoxy) benzaldehyde in ethanol at 5°C catalysed by sodium hydroxide. The crude product 1-(2-nitroethenyl)-4-(phenylmethoxy)benzene (*Compound 19*) was recrystallised from ethanol in good yield, (*Scheme 2.39*). ^1H NMR Spectroscopy

corroborated the structure of the product with two doublets corresponding to the alkene protons at δ 7.43 and δ 7.85 ppm. The latter peak at δ 7.85 is more deshielded than the former and as a result it is likely that this peak can be assigned to the olefin proton nearest the aryl group.

Reduction of *Compound 19* may be accomplished by catalytic hydrogenation or by the use of chemical reducing agents in acidic solution. The nitro compounds readily undergo reduction to yield the primary amines and this method constitutes the most general synthesis of aromatic amines.



Compound 19.

Scheme 2.39.

2-(4-(Phenyl-methoxy)-phenyl)-ethyl-amine was prepared in a Soxhlet apparatus using an excess of lithium aluminium hydride ($LiAlH_4$) which was boiled under reflux in dry ether as the nitro compound was added dropwise from a side funnel. The reflux was continued for 16 hours before the $LiAlH_4$ was quenched with water and the product (*Compound 20*) was extracted in a conventional work-up, (*Scheme 2.40*).

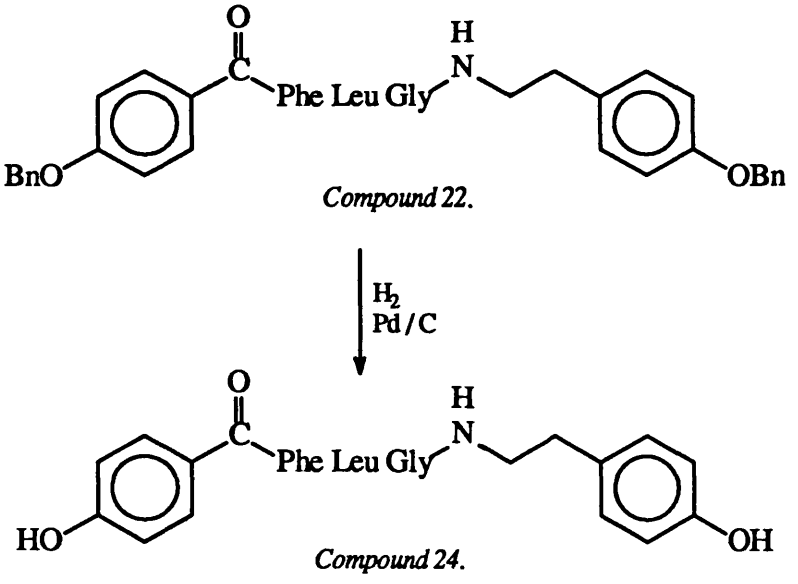
Compound 20.

Scheme 2.40.

The primary amine was not detected by ^1H NMR spectroscopy, however, the four protons

comprising of the carbon chain terminating in the amine were assigned as triplets at δ 2.56 and δ 2.71 in the spectrum.

The successful synthesis of three bis(benzyloxy)phenyl protected model compounds containing di-, tri-, and tetra- peptide sequences (GlyGly (*Compound 21*), PheLeuGly (*Compound 22*) and GlyPheLeuGly (*Compound 23*) respectively) is described fully in Chapter 3. In an attempt to cleave the phenolic protecting groups to allow further derivatisation of the diol compounds the bis(benzyloxy)phenyl groups were hydrogenated in ethanol using a palladium over charcoal catalyst and a hydrogen atmosphere, (*Scheme 2.41*).



Scheme 2.41.

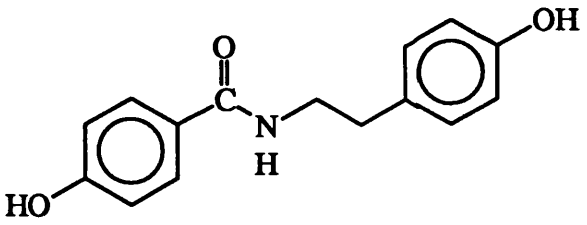
Conventional work-up of the tripeptide, PheLeuGly, (*Compound 22*) furnished a material that was isolated in good yield and identified as the diol, (*Compound 24*). A spectrum generated by ^{1}H NMR was assigned accurately to the proposed structure of the compound with two characteristic singlets at δ 9.22 and δ 10.06 providing further evidence of the newly formed phenolic groups. Further characterisation was provided by mass

spectroscopy with the observation of the M + H ion at *m/z* 575 and *m/z* 573 in the FAB [+] and FAB [-] mass spectrum.

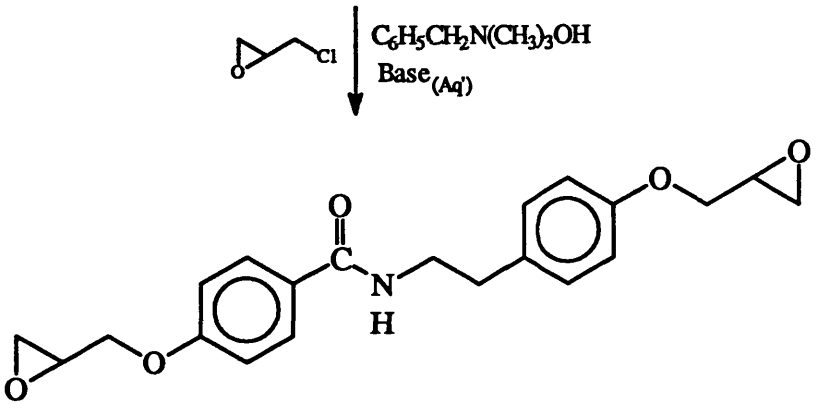
Hydrogenation of the bis(benzyloxy)phenyl tetrapeptide (*Compound 23*) in a similar experimental technique to the above afforded a product in good yield. Unfortunately, characterisation of this material by ^{1}H NMR and subsequently by COSY provided no evidence that could confirm the identity of this material. A further attempt to characterise this material with FAB [-] mass spectoscopy confirmed the prescence of the required product (*Compound 25*) with a M - H ion at *m/z* 630.

2.6. Epichlorohydrin experiments

In Section 2.4.3., the successful demethylation of test *Compound 12*, 4-methoxy N-(2-(4-methoxyphenyl)ethylbenzamide) resulted in a bis(hydroxy) compound (*Compound 14*) that was suitable for further reaction with epichlorohydrin. *Compound 14* was an extremely important analogue to the latter compounds (*Compound 24* and *Compound 25*), which were derived from an alternative strategy, but possessed identical functional groups. As a model, the diol (*Compound 14*) was dissolved in aqueous base with an excess of epichlorohydrin and benzyltrimethylammonium hydroxide (a phase transfer catalyst). Conventional work-up gave a multicomponent gum but direct application to a chromatography column and subsequent elution permitted the isolation of a single product in moderate yield. This material was identified as the bis(glycidylether), (*Compound 80*) by accurate analysis of the elemental composition and mass spectroscopy. Further corroboration, by proton spectroscopy revealed no evidence of the phenolic signals and a cluster of peaks *circa* δ 2.71 - 4.39 could be assigned to the bis(glycidylether), (*Scheme 2.42*).



Compound 14.

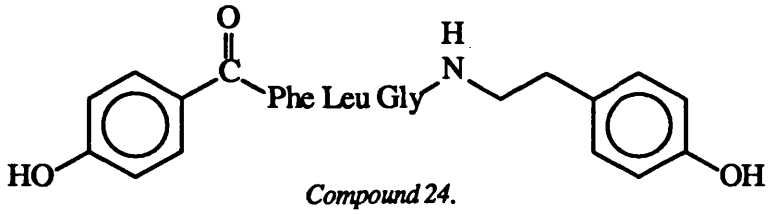


Compound 80. *Scheme 2.42.*

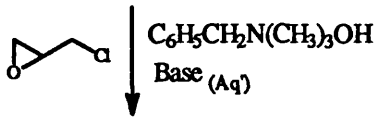
The success of this experiment was tremendously exciting as it opened up a synthetic pathway *via* the activated PEGs to the target polymerisation reactions.

The conversion of the oligopeptide diols to oxiranyl compounds involved a similar experimental strategy to the one described above. In the case of the tripeptide, PheLeuGly, (*Compound 24*) removal of the organic solvent by evaporation afforded a multicomponent material that was separated by column chromatography. Elution of a single product in good yield led to characterisation studies which confirmed the identity of the bis(glycidylether)tripeptide, (*Compound 79*) (*Scheme 2.43*). Proton spectroscopy revealed a "crowded" spectrum but comparison of the integrals of the leucyl methyls at δ 0.84 and 0.88, of the oxiranyl cluster *circa* δ 2.69 - 3.30 and of the aromatic cluster δ 7.00 - 7.79 showed a 1 : 1 ratio of bis(glycidylether) to tripeptide. Further elucidation of the spectrum using COSY enabled each signal to be assigned more accurately, (*see Spectrum I, Appendix 1*). Mass spectroscopy provided additional evidence as to the identity of the

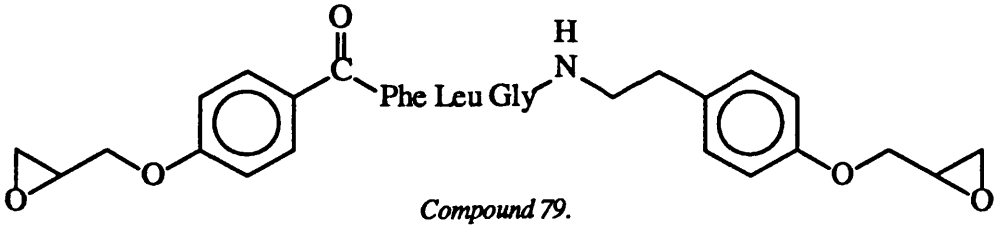
product with the observation of the M ion at *m/z* 687 with FAB [+] and at 684 for FAB [-].



Compound 24.

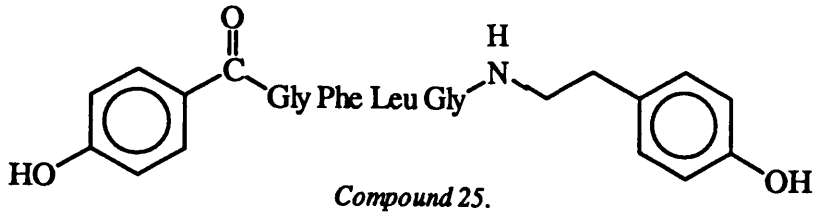


Scheme 2.43.

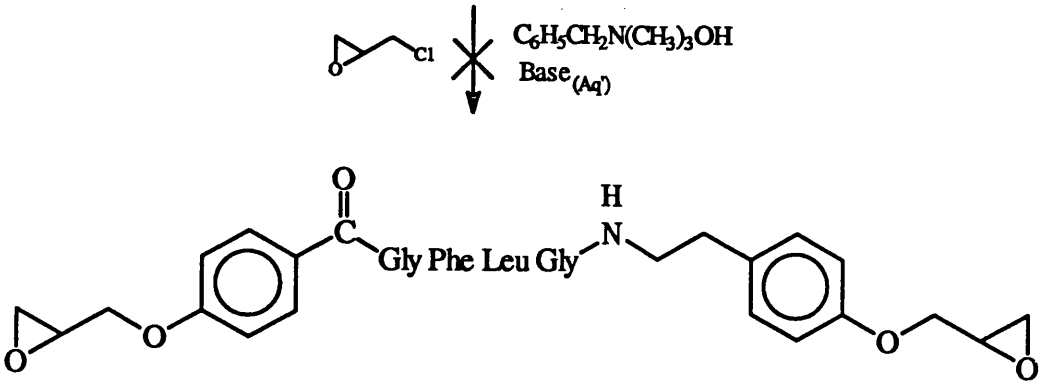


Compound 79.

Epoxidation of the tetrapeptide GlyPheLeuGly (*Compound 25*) was attempted in order to synthesise the target bis(glycidylether) compound. However, treatment of the diol with an excess of epichlorohydrin and phase transfer catalyst gave no material which could be identified as the required bis(epoxide) derivative, (*Scheme 2.44*).



Compound 25.



Scheme 2.44.

Chapter 3. Peptides.

3.1. Strategy.

It is proposed that polymerisation of PEG with alternating oligopeptide sequences would afford a biodegradable copolymer. The peptide sequences are to be selected for their sensitivity to hydrolysis by lysosomal enzymes. The synthesis of a library of oligopeptide analogues, *i.e.* GlyPheLeuGly, GlyGly, GlyPheGly, PheLeuGly, and Gly is to be pursued in order to compare the rates of polymer degradation with lysosomal enzymes. In addition amino acids containing side chains, *e.g.* glutamic acid could be incorporated into the copolymer to provide pendant linkages which may be further activated in order to attach drugs or targeting groups.

The oligopeptides were prepared using standard solution coupling methods. For example, 2-(4-(benzyloxyphenyl)ethylamine was coupled to BOCglycine using DCC. Treatment with HCl afforded the deprotected amine salt. Coupling of this amine with BOC protected amino acid and dipeptide active esters served to extend the peptide chain in an iterative process.

3.2. Introduction.

The L-amino acids constitute a particularly important class of natural di-functional compounds because they are the building blocks from which proteins are constructed. They all have the general structure given in *Figure 3.1(A)*, or in the case of the atypical amino acid, L-proline, *Figure 3.1(B)*.

(A). (B).

Fig. 3.1.

The amino acids, in part, owe their importance to the fact that they may form amide or peptide bonds which link these building blocks together forming natural polymers or proteins. Their fascination is extended further by the fact that each of the twenty members of the amino acid series have a unique chemical identity. This is reflected in proteins which may be acidic, basic or neutral; globular, soluble or insoluble, depending on their amino acid composition. It follows that there has been an enormous level of research into clinical applications for these biomaterials, both as mimics of endogenous ligands for biochemical pathways, or as peptide drugs.

Peptides (which are classed as molecules containing fewer than fifty amino acids) are often 'tailor made' to conform to specific criteria, *e.g.* size or solubility constraints. Oligopeptides containing amino acids such as lysine or glutamine, which possess side chains terminating in functional groups, are able to undergo further chemical modification.

In the early 1980s, a plethora of papers were published on the design of short oligopeptide chains that would match known specificities of lysosomal enzymes. By varying the peptide length and composition, the susceptibility to lysosomal hydrolysis under different conditions of pH or enzyme sub-type could be controlled. Rejmanová *et al* (138) optimised an amino acid sequence which facilitated the intracellular degradation by lysosomal thiol proteases. The oligopeptide consisted of

four amino acids, Gly-Phe-Leu-Gly, which has been extensively studied over the last ten years for use as a labile drug-polymer linkage.

Oligopeptides can be synthesised in a stepwise assembly controlled through a series of sequential protection, coupling and deprotection reactions. Protecting groups have been developed for both the amino and carboxy groups as well as for other groups that occur in the side chains of the various amino acids. A suitable protecting group must fulfil several criteria:

1. The protecting group should be easy to introduce into the molecule;
2. The protecting group should not adversely affect the molecule;
3. The protecting group should protect the functional group under conditions of amide formation;
4. The protecting group must be removable, under conditions that have no adverse effect on the structure being assembled;
5. The protecting group should be susceptible to specific cleavage which does not affect other protecting groups on the molecule;
6. The protecting group should not, at any stage, jeopardise the chiral integrity of nearby chiral centres.

3.3. α-Amino protection.

The groups that are available for α-amino group protection can be divided into five categories; the alkoxycarbonyl groups, triphenylmethyl (trityl) protection, 2-nitrophenylsulphenyl protection (Nps), diathiasuccinoyl (Dts) and diphenylphosphinyl (Dpp) protection.

3.3.1. Alkoxycarbonyl Groups.

The alkoxycarbonyl family of amino protecting groups has dominated the field of amino-protection for many years, mainly due to their stability, selective ease of removal, and chiral integrity.

3.3.1.1. Benzyloxycarbonyl (CBZ) protection.

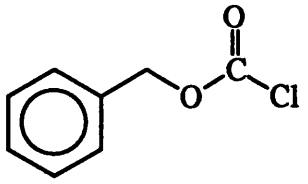


Fig. 3.2.

The CBZ group is normally introduced *via* the chloroformate reagent, (*Figure 3.2*) (330), but minor side reactions such as dipeptide formation under Schotten-Baumann conditions with amino acids are occasionally a nuisance. A less reactive acylating agent is often preferred, (*Figure 3.3*).

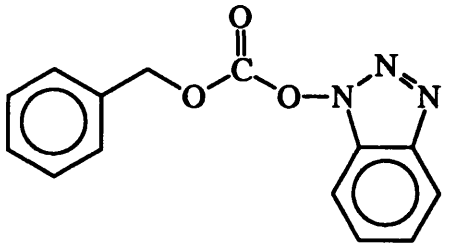
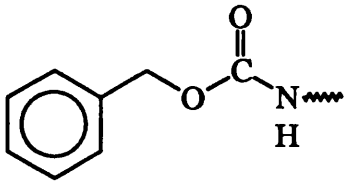


Fig. 3.3.



The cleavage conditions of the CBZ group include hydrogen bromide (HBr) / protic acid (AcOH), Lewis acid (trimethylsilyl iodide), or catalytic hydrogenolysis. However, it should be noted that the prescence of a good nucleophile is important for the former two cleavages. This protecting group is stable under moderately basic

or non-nucleophilic acidic conditions (TFA), enabling it to be carried through a range of structural intermediates. Electron-releasing substituents in the aromatic ring accelerate the cleavage, whereas electron-withdrawing groups have the opposite effect. For instance, the 4-methoxybenzyloxycarbonyl group which is cleaved by TFA, is the most useful ring-substituted analogue (235), (*Figure 3.4*).

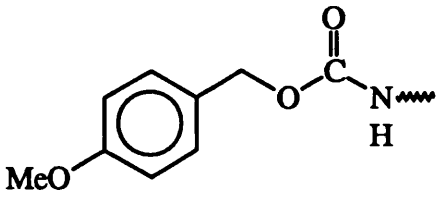


Fig. 3.4.

3.3.1.2. t-Butoxycarbonyl (BOC) protection.

The BOC anhydride is a convenient solid which gives good yields and can be stored under refrigeration for long periods (236).

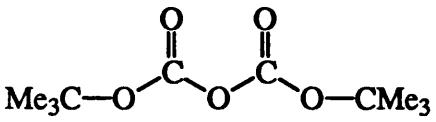
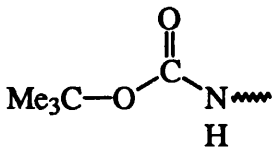


Fig. 3.5.



The BOC group (*Figure 3.5*) is completely stable to catalytic hydrogenolysis conditions, but it is much more labile to acids than the CBZ group, to which it is therefore completely orthogonal. BOC removal is conveniently carried out by dissolution in TFA, however mineral acids will attack BOC groups rapidly and must be avoided in wash procedures. Basic and nucleophilic reagents have no effect on the BOC group, even on prolonged exposure. In this respect the stability of BOC is better than that of the CBZ groups.

3.3.1.3. 2-(4-Biphenyl)-isopropoxycarbonyl (Bpoc) protection.

The Bpoc group (*Figure 3.6*) (237) is even more acid labile than BOC, and can be removed by brief treatment with, for example, chloroacetic acid / dichloromethane mixtures at ambient temperature. These are conditions which would leave BOC and CBZ groups intact. Bpoc is very stable to bases and nucleophiles, but like the CBZ group, it is cleaved by catalytic hydrogenolysis.

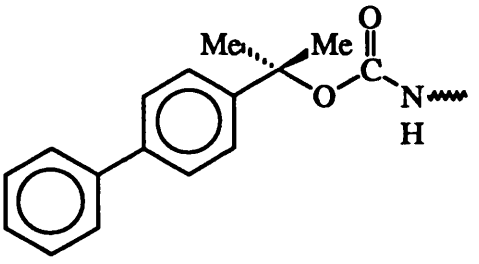


Fig. 3.6.

The Ddz (α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl) (*Figure 3.7*) group is comparable to Bpoc in their lability to acids; it is also removed by photolysis.

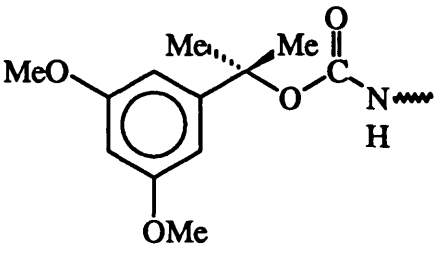


Fig. 3.7.

3.3.1.4. 9-fluorenylmethoxycarbonyl (Fmoc) protection.

The Fmoc group, according to Carpino (238) is normally introduced in the Schotten-Baumann manner, using the rather stable chloroformate, (*Figure 3.8*) although several less reactive reagents have been investigated, *e.g.* the succinimido carbonate.

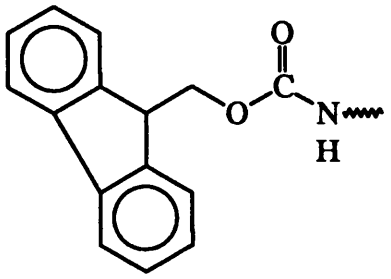


Fig. 3.8.

The Fmoc group is very stable to acidic reagents, but it is cleaved swiftly under certain basic conditions. Piperidine is the routine reagent but other systems, *e.g.* fluoride ion in DMF are also effective.

3.3.1.5. 2,2,2-Trichloroethoxycarbonyl (Troc) protection.

The Troc group (*Figure 3.9*) is stable to both acid and base, but Woodward reported that it is smoothly cleaved by zinc dust in acetic acid (239).

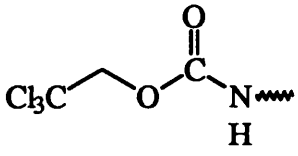


Fig. 3.9.

3.3.2. Triphenylmethyl (trityl, Trt) protection.

The principal effectiveness of the trityl protecting group is largely dependent on steric hindrance, (*Figure 3.10*).

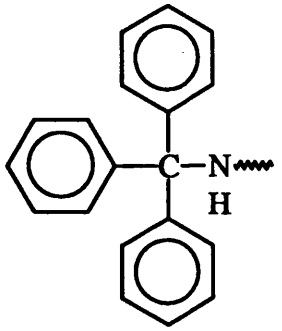


Fig. 3.10.

The trityl group is very stable to base and very labile to acid. It does not survive even mildly acidic conditions which is advantageous since it enables the use of gentle deprotection reagents such as acetic acid, and rarely catalytic hydrogenation (240).

3.3.3. 2-nitrophenylsulphenyl (Nps) protection.

Zervas prepared Nps amino acids by the reaction of NpsCl with amino acids in basic conditions (241). They are not very stable, and are generally isolated, purified and stored as dicyclohexylammonium salts from which they can be liberated when required by acidification with sulphuric acid, (*Figure 3.11*).

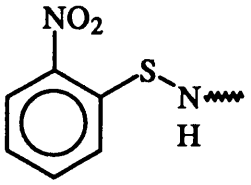


Fig. 3.11.

The Nps group is stable to mild base, but not to acidic conditions (HCl) or to catalytic hydrogenolysis.

3.3.4. Dithiasuccinoyl (Dts) protection.

The Dts protected amino acids, (*Figure 3.12*) are not widely employed perhaps as a result of their complicated synthesis *via* esters (242). However, this group is completely orthogonal to BOC and other acid-labile groups, as cleavage is induced by base-catalysed disulphide exchange with thiols.

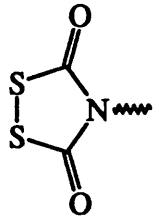


Fig. 3.12.

3.3.5. Diphenylphosphinyl (Dpp) protection.

Kenner reported that Dpp amino acids, (*Figure 3.13*) are prepared indirectly *via* esters using diphenylphosphinyl chloride (243). The Dpp group survives both basic and catalytic hydrogenolysis, but is cleaved by mild acids, *e.g.* TFA.

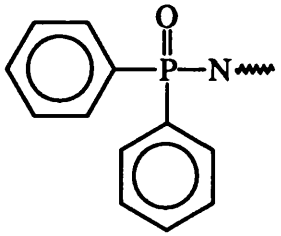


Fig. 3.13.

3.4. α-Carboxy protection.

The usual means of carboxy protection is esterification, and seven types are discussed more fully.

3.4.1. Methyl and ethyl esters.

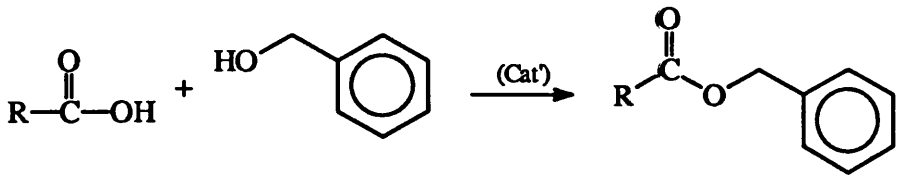
Methyl and ethyl esters provide good carboxy protection throughout most peptide bond forming procedures and deprotective operations. Their removal, however, often calls for rather vigorous treatment involving hydrolysis, which may cause racemisation.

Amino acids react easily with hot methanolic hydrogen chloride to give the corresponding methyl ester hydrochlorides. Treatment with thionyl chloride and methanol is however, a convenient alternative procedure (244), (*Scheme 3.1*).

Scheme 3.1.

3.4.2. Benzyl esters.

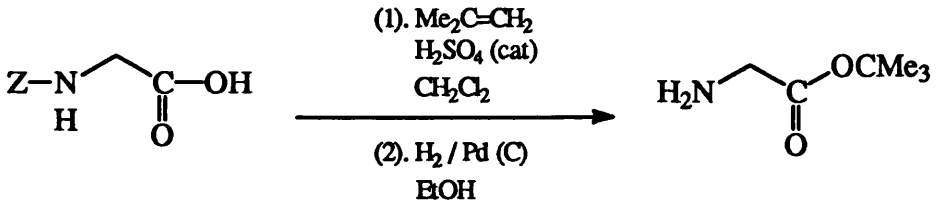
Amino acid benzyl esters (245) are best prepared by 4-toluenesulphonic acid catalysed esterification with benzyl alcohol, driven by azeotropic removal of water (*Scheme 3.2*). Benzyl ester groups are cleaved by hydrolysis and by hydrazinolysis. More importantly, they are cleaved by HBr /AcOH, HF and catalytic hydrogenolysis.



Scheme 3.2.

3.4.3. *t*-Butyl esters.

Unlike methyl and benzyl esters, amino acid *t*-butyl esters are stable in the free base form and diketopiperazines (DKPs) are not easily formed from the dipeptides. They can be prepared directly from the amino acids, but the standard procedure is indirect, (*Scheme 3.3*).



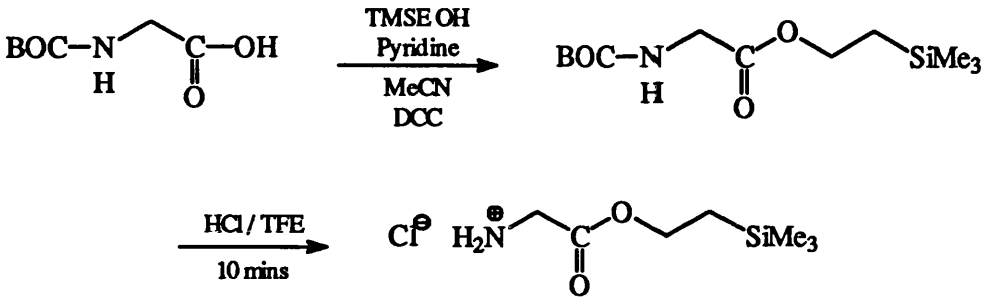
Scheme 3.3.

Alternatively:- BOC F Bu^tOH
Et_3N DMAP
CH_2Cl_2

The stability and lability of *t*-butyl esters generally parallel the properties of the BOC group.

3.4.4. 2-Trimethylsilylethyl esters (Tmse).

The Tmse group (*Scheme 3.4*), is orthogonal to acid-sensitive groups (245). Tmse survives catalytic hydrogenation conditions and very mild acid, but is cleaved rapidly with TFA. However, the preferred method of cleavage is with quarternary ammonium fluoride in DMF, which often proceeds smoothly.



Scheme 3.4.

3.4.5. Allyl esters.

Allyl esters, (*Figure 3.14*) can be cleaved under the influence of palladium complexes without disturbing BOC, CBZ, Fmoc or related groups.

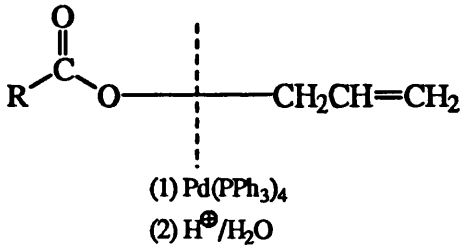
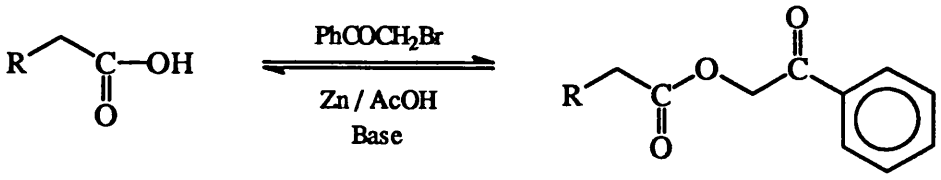


Fig. 3.14.

3.4.6. Phenacyl esters (Pac).



Scheme 3.5.

Phenacyl ester (Pac) protection, (*Scheme 3.5*) is very stable towards acidolytic conditions. It is not stable to catalytic hydrogenolysis, but complete orthogonality with α-BOC protection makes it a useful reagent.

3.5. The Synthesis of the Enzyme Labile Peptide Sequence, Gly Phe Leu Gly.

The work discussed in this section concerns the strategy adopted for the complete synthesis of the tetrapeptide Gly-Phe-Leu-Gly, within the chosen aryl side groups. In addition to the enzyme labile peptide sequence other oligopeptide derivatives were synthesised which, hypothetically, do not undergo lysosomal degradation.

The strategy adopted for peptide chain synthesis was often divergent in that the amino acids were first joined into dipeptide fragments of aryl conjugates and these were then allowed to react. The advantages of "fragment condensation" over the "stepwise" approach include an increase in overall yield, better isolation of products and reduction in synthesis time. On the other hand, the choice of protective group was often made more complicated and racemisation can be a problem.

The remainder of this chapter can be divided into three sections. Section 3.5.1. is an account of the oligopeptide chains synthesised in connection with the 4-methoxy(phenyl) derivatives. An alternative strategy, in Section 3.5.2., focuses on the 4-phenylmethoxy(phenyl) peptidic compounds which were precursors to the attachment of epoxides. Finally, in Section 3.5.3, there is a brief discussion on the synthesis of oligopeptide chains bearing side chains for further modification.

3.5.1. 4-Methoxy(phenyl) derivatives.

The target compound was N-(N-(N-(N-(4-methoxybenzoyl)glycyl)-phenylalanyl)leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide, (*Figure 3.15*).

Preliminary experiments therefore addressed the feasibility of introducing a single amino acid onto the aryl functional groups with glycine.

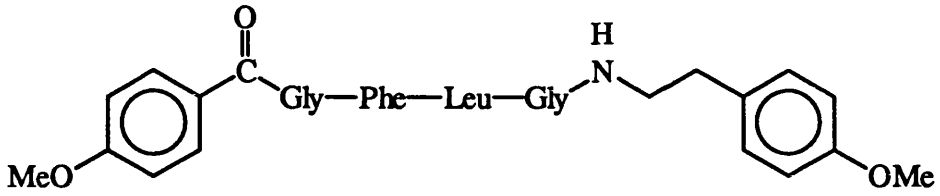
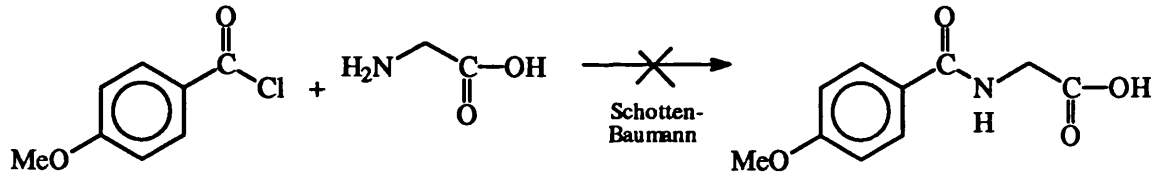


Fig. 3.15.

3.5.1.1. The Mono Amino Acid Compounds. (Gly).

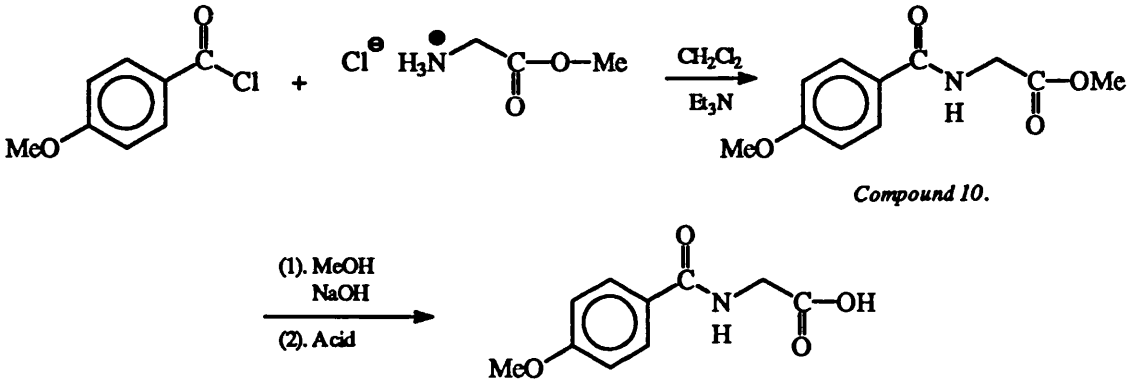
Gledhill *et al* (246) prepared many N-benzoylglycines by conventional Schotten-Baumann techniques by addition of the aroyl chloride to the amino acid in aqueous sodium hydroxide solution, followed by acidification and collection of the N-aryl amino acid by filtration. Despite previous reports with related compounds (247) numerous attempts to repeat the Schotten-Baumann technique using 4-methoxybenzoyl chloride and glycine proved difficult and multi-component materials were afforded which could not be identified as the required N-aryl glycine, (*Scheme 3.6*).



Scheme 3.6.

The carboxy-protected glycine methyl ester is more soluble in organic solvents than glycine and an alternative strategy was undertaken. 4-Methoxybenzoyl chloride was carefully added to glycine methyl ester hydrochloride in dichloromethane containing triethylamine (a tertiary amine base). Conventional work-up afforded a product in good yield which was characterised fully, (*Scheme 3.7*). Examination of the mass spectral fragmentation pattern

confirmed N-(4-methoxybenzoyl)glycine methyl ester, *Compound 10* with a peak at *m/z* 223 M + H. Data from elemental analysis and ^{1}H NMR is consistent with the identity of *Compound 10.*



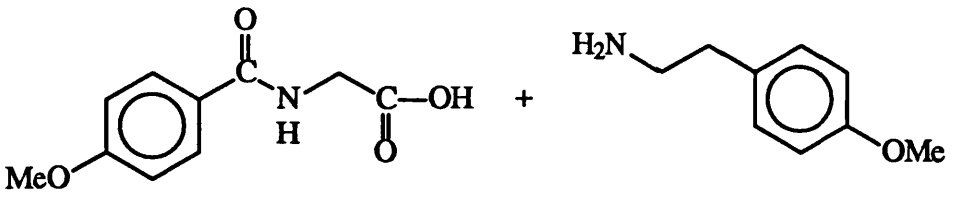
Compound 10.

Compound 26.

Scheme 3.7.

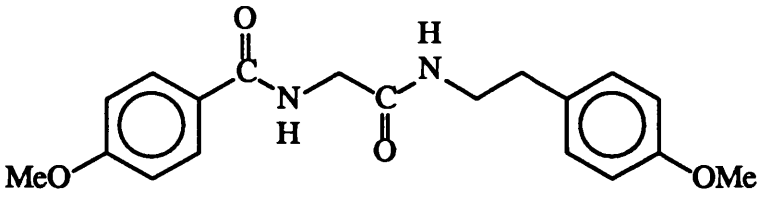
Deprotection of the methyl ester in boiling methanolic sodium hydroxide was straightforward and following acidification, *Compound 26* was produced in good yield. The identity of the product was shown by ^{1}H NMR which revealed the disappearance of the methyl ester at δ 3.81, which is consistent with *Compound 26.*

Reaction of *Compound 26* and N-(2-(4-methoxyphenyl)ethyl)amine using DCC as a coupling reagent can be achieved, but only in moderate yield, (*Compound 27*), (*Scheme 3.8*).



Compound 26.

Scheme 3.8.



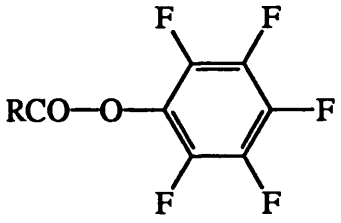
Compound 27.
(Moderate Yield)

It was therefore appropriate to use an activating agent which would facilitate the attack of the amino group on the acyl carbon on the carboxy component. Active esters are suitable activating agents and include 4-nitrophenyl ester (ONp) [*Fig. 3.16(a)*], penta and trichlorophenyl ester (OPCP, OTCP) [*Fig. 3.16(b)*], pentafluorophenyl ester (OPfp) [*Fig. 3.16(c)*], and succinimido ester (OSu) [*Fig. 3.16(d)*].

(A).

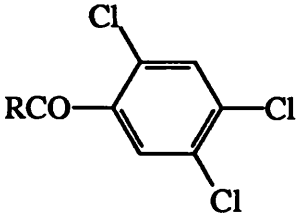
RCO_2Np

(C).



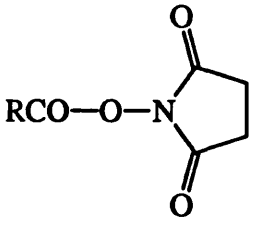
RCO_2Pfp

(B).



RCO_2Tcp

(D).

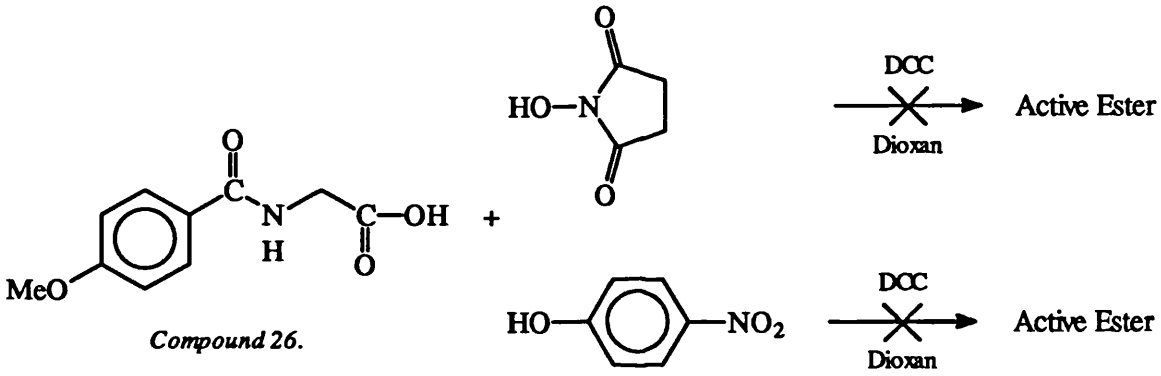


RCO_2Su

Fig. 3.16.

Attempts were made to prepare three active ester derivatives of N-(4-(methoxy-benzoyl)glycine (*Compound 26*), namely the 4-nitrophenyl ester (ONp), succinimido ester (OSu) and pentafluorophenyl ester (Pfp). These derivatives were prepared by a common route described by Atherton (248) which involved DCC mediated coupling in dioxan at 0°C.

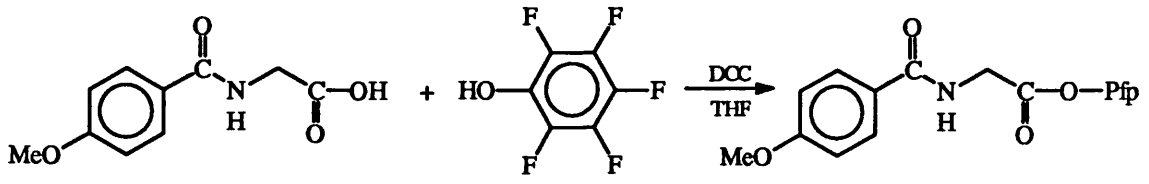
Treatment of the carboxy component with 4-nitrophenol or hydroxysuccinimide resulted in intractable mixtures of products and starting material which were not purified any further, (*Scheme 3.9*).



Compound 26.

Scheme 3.9.

On the contrary, esterification with pentafluorophenol at 0°C in dry THF was successful, (*Scheme 3.10*). The by-product dicyclohexylurea (DCU) was removed and a conventional work-up gave pentafluorophenyl ester in good yield, (*Compound 28*).

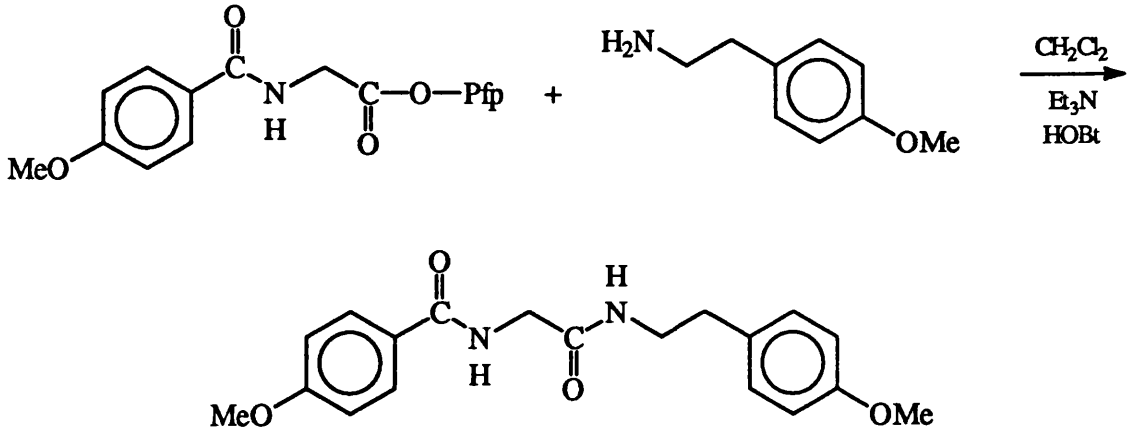


Compound 26. *Compound 28.*

Scheme 3.10.

The disadvantage of using an active ester such as Pfp over Np, OSu or TCP is that it is essentially invisible to ^1H NMR detection. However, the influence of this electron-withdrawing group was reflected in the shift change of the glycyl CH_2, which was more deshielded in *Compound 28* (δ 4.57) than *Compound 26* (δ 3.92). Fluorine spectroscopy, ^{19}F NMR, confirmed the integrity of the pentafluorophenyl group and additional evidence to the identity of the product was provided by mass spectroscopy (*m/z* 376) (M + H) and elemental analysis.

Since N-(4-methoxybenzoyl)glycine pentafluorophenyl ester (*Compound 28)* should be a good electrophile, synthesis of N-(4-methoxybenzoyl)glycine-N-(2-(4-methoxyphenyl)-ethyl)amide was reinvestigated. The active ester was treated with the primary amine in the presence of triethylamine (a tertiary amine base), (*Scheme 3.11*). The product was isolated in good yield and identified as *Compound 27*. Comparison of the integrals of the aromatic methoxy singlets at δ 3.70 and δ 3.81 and the amide multiplet at δ 7.85, verified the structure. Further characterisation by mass spectroscopy confirmed the identity of the material with the M + H ion at *m/z* 343.

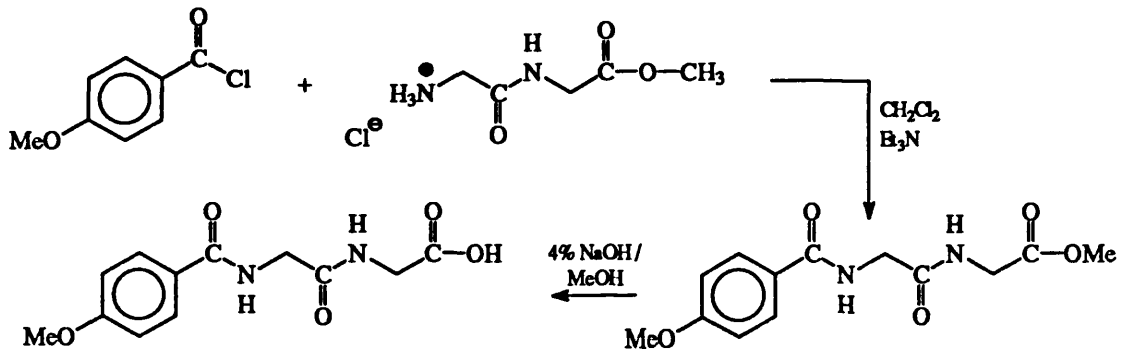


Compound 27.

Scheme 3.11.

3.5.1.2. The Dipeptide Compounds. (GlyGly)

The task of synthesising the dipeptide GlyGly within the model aryl side groups appears deceptively simple. A number of strategies were explored. A useful route to the dipeptide would come from the reaction of 4-methoxybenzoyl chloride with a dipeptidemethyl ester hydrochloride. An attempt was made to treat 4-methoxybenzoyl chloride with N-glycylglycine methyl (*Scheme 3.12*) and ethyl esters in the presence of triethylamine in a similar experimental protocol to *Compound 10*.

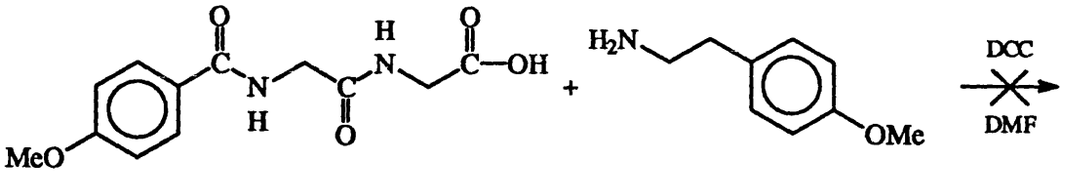


Compound 31. *Compound 29.*

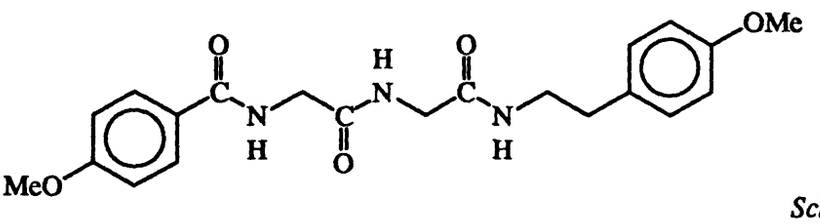
Scheme 3.12.

4-Methoxybenzoyl GlyGly methyl ester (*Compound 29*) was isolated in good yield and identified by analytical techniques. The newly-formed amide peak at δ 8.69 was identified in the ^1H NMR spectrum along with the aryl methoxy group at δ 3.83 and the ester methyl group at δ 3.65. This characterisation was substantiated by mass spectroscopy and elemental composition data. Synthesis of the 4-methoxybenzoyl GlyGly ethyl ester (*Compound 30*) was straightforward with the glycyl ethyl ester group identified at δ 1.18 (3H) and δ 4.08 (2H) in the ^1H NMR spectrum. The facile removal of the ester groups in a hydrolysis step gave products which were shown, by ^1H NMR characterisation, to be the deprotected dipeptide, (*Compound 31*).

Several experiments were conducted to investigate the feasibility of coupling *Compound 31* directly with N-(2-(4-methoxyphenyl)ethyl)amine in a DCC reaction to give the desired target compound, (*Scheme 3.13*).

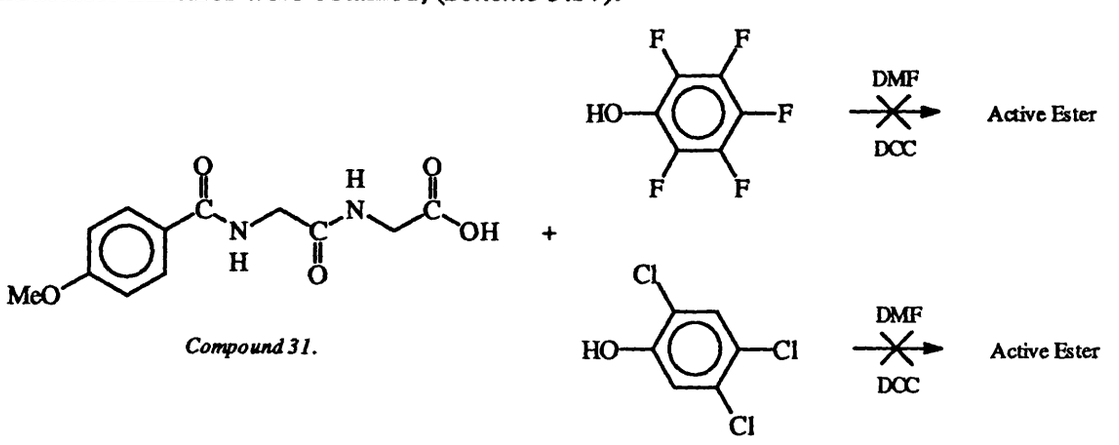


Compound 31.



Scheme 3.13.

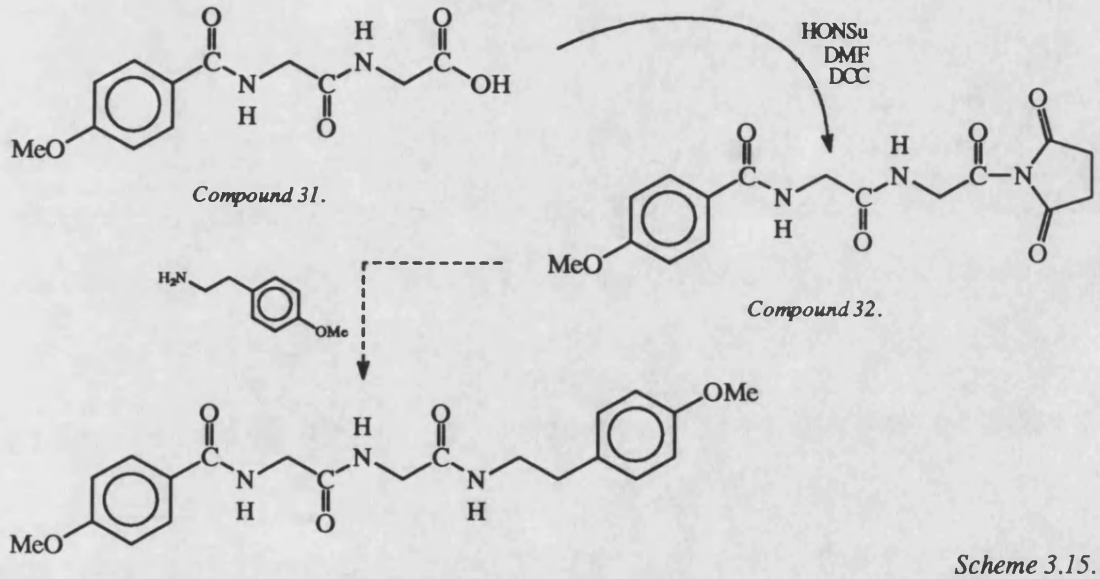
Unfortunately, this method gave no material which could be identified as the required compound and a more indirect approach was sought. Attempted esterification of *Compound 31* with pentafluorophenol or 2,4,5-trichlorophenol was unsuccessful and intractable mixtures were obtained, (*Scheme 3.14*).



Compound 31.

Scheme 3.14.

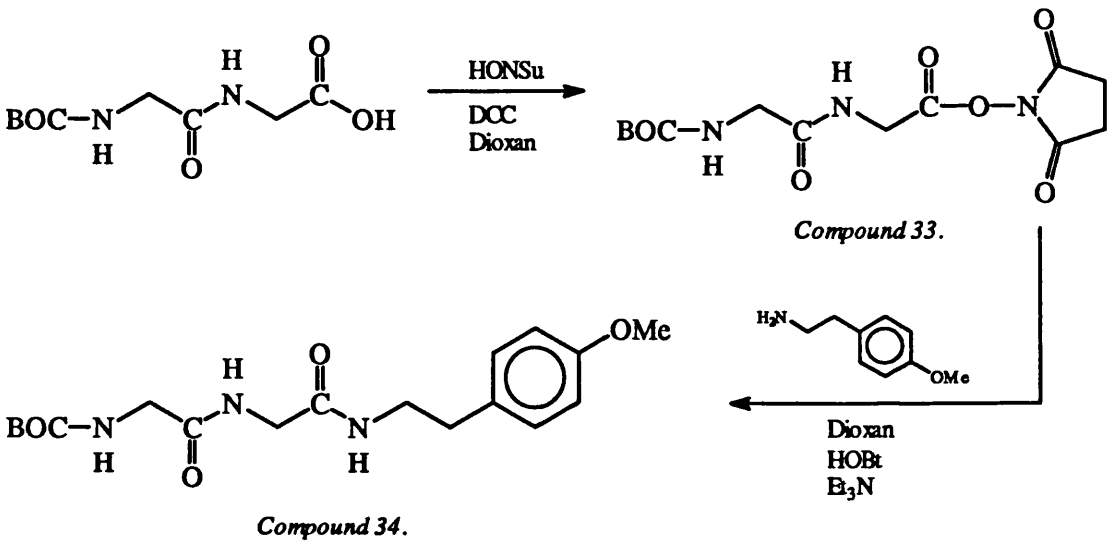
A related reaction with N-hydroxysuccinimide and DCC was investigated in DMF at 0°C, (*Scheme 3.15*). The removal of the DCU and DMF afforded a crude material which was recrystallised and a single product was isolated. The identity of the active ester of the dipeptide was confirmed by ^{1}H NMR spectroscopy as *Compound 32*. The logical succession to this experiment was the reaction of *Compound 32* with N-(2-(4-methoxy-phenyl)ethyl)amine, however this work was not completed.



Scheme 3.15.

Several experiments were conducted to investigate whether a α-amino protected dipeptide (BOCGlyGly) could be encouraged to undergo a coupling reaction with N-(2-(4-methoxyphenyl)ethyl)amine. Preliminary experiments using DCC for a direct coupling were only partially successful. A crude product was afforded after a conventional work-up, which was identified as a mixture of two components by ^{1}H NMR. The ratio of the two components was calculated by comparison of the integrals, and found to be approximately 2:1 in favour of the desired product. Isolation of the two compounds by column chromatography recovered the uncontaminated product in poor yield.

In contrast, an alternative strategy was attempted whereby the dipeptide, N-glycylglycine was α-amino protected by BOC, in the conventional manner, (249) and the acyl peptide ester was prepared, in good yield, by the reaction of N-hydroxysuccinimide with equivalent amounts of DCC and *Compound 33*, (*Scheme 3.16*).



Compound 34.

Scheme 3.16.

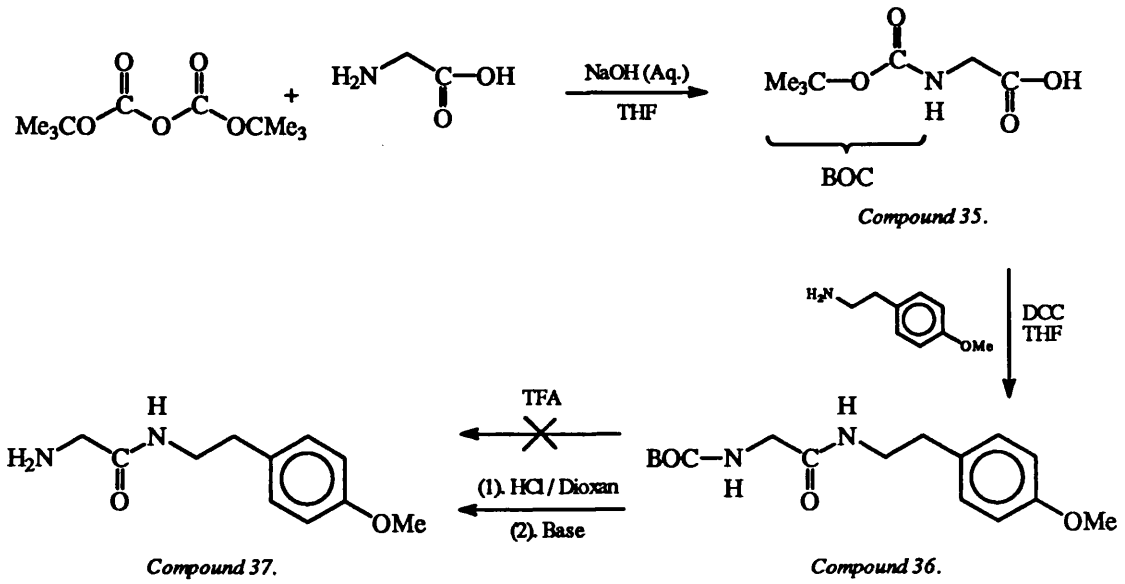
Nucleophilic substitution of the active ester by N-(2-(4-methoxyphenyl)ethyl)amine was carried out in a catalysed reaction at elevated temperature. Following a conventional work-up, a product was isolated in a moderate yield. Evidence as to the identity of the product was provided by ^{1}H NMR. Comparison of the large singlet at δ 1.44, of the three amide NH signals at δ 5.49, 6.70 and 7.19 and of the aromatic cluster at δ 6.82 - 7.09, showed a 1 : 1 ratio of BOCGlyGly to the aryl side group, (*Compound 34*). One may speculate that the deprotection of *Compound 34* to the hydrochloride salt would provide a suitable intermediate with which 4-methoxybenzoyl chloride could be treated, and the target compound formed. However, this experiment was not performed as the

disadvantage with this hypothesis lies in the fact that previous attempts to treat the acid chloride with a primary amine have met with disappointing results.

A simpler option was to generate the dipeptide in the final coupling reaction, as opposed to carrying the dipeptide through a synthetic pathway.

Glycine was successfully amino-protected under basic conditions with di-*t*-butyl dicarbonate [$(BOC)_2O$] according to the method of Anderson and McGregor (249). The crystalline product, (*Compound 35*) was obtained in good yield and the literature melting point was confirmed.

Further reaction of BOC-glycine with 2-(4-methoxyphenyl)ethylamine was completed by mixing the amino and carboxy components with dicyclohexylcarbodiimide (DCC) in equimolar amounts in tetrahydrofuran. After 16 hours at 5°C, the DCU was filtered off and the product was afforded in good yield following recrystallisation, (*Scheme 3.17*). *Compound 36* was often obtained in better yield if a slight excess of BOC-Gly was used. Characterisation as *Compound 36* was achieved by ^1H NMR.



Compound 35.

Compound 37.

Compound 36.

Scheme 3.17.

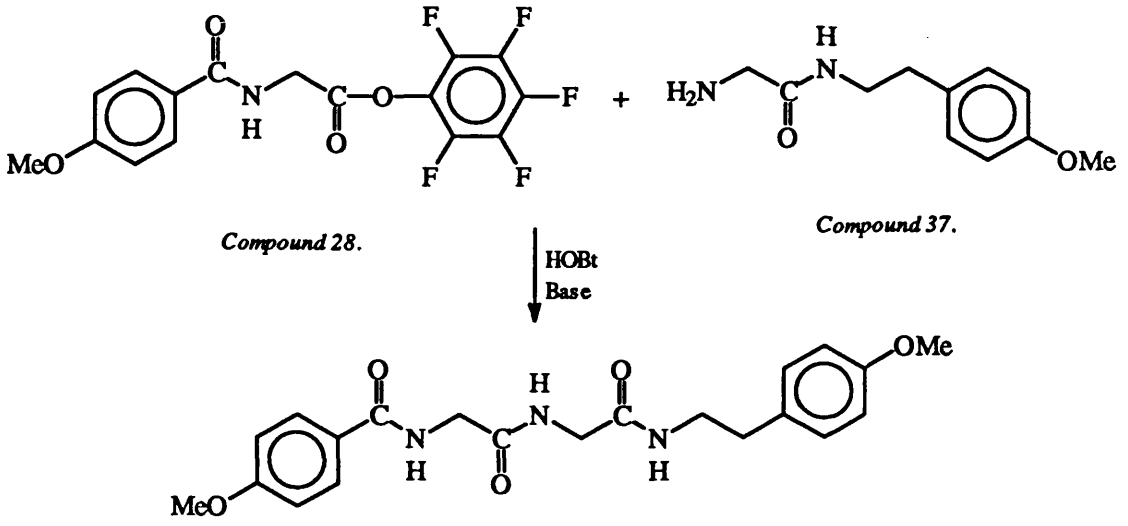
The 1:1 ratio of the singlet peaks of the *t*-butyl group at δ 1.37 and of the 4-methoxy group at δ 3.72 suggested the coupling reaction had been successful. This was corroborated by the broad triplet at δ 6.91 which corresponds to the newly formed amide. This verdict was confirmed by observation of the M + H ion at *m/z 309* in the mass spectrum and through elemental analysis.

Treatment of *Compound 36* in an acid milieu enabled the protecting group to be cleaved. An attempt to remove BOC with a large excess of TFA failed to give any material which could be identified as the deprotected compound. Further investigation using hydrogen chloride was successful and the free base, (*Compound 37*) was given up by treatment with aqueous sodium hydroxide. Verification by ^{1}H NMR clearly showed that the large singlet at δ 1.37 corresponding to the BOC group had been removed and a primary amine, δ 8.30, had been formed.

The flexibility of the divergent strategy adopted for the synthesis of the target compound enabled further model compounds to be developed for attachment of epoxides.

Previous experiments had established the synthesis of N-(4-methoxybenzoyl)-glycine pentafluorophenyl ester (*Compound 28*) and glycine N-(2-(4-methoxyphenyl)-ethyl)amide (*Compound 37*). An attempt to extend the scope of these intermediates involved reaction of the active ester of *Compound 28* with the primary amine of *Compound 37* in ethyl acetate in the presence of a catalytic amount of HOBt. Recrystallisation permitted the isolation of a single product in modest yield, (*Scheme 3.18*). This material was identified as the dipeptide target compound by standard analytical techniques, (*Compound 38*). The M + H ion at *m/z* 400 and M - H ion at *m/z* 398 were observed in the mass spectrum for FAB [+] and FAB [-] respectively. Further

verification of the identity by ^1H NMR spectroscopy revealed singlet peaks at δ 3.70 and δ 3.81 for the aromatic methoxy group which were in agreement with the integrated glycyl doublet protons at δ 3.23, 3.67 and glycyl amides; δ 8.18 and 8.73.



Compound 28. *Compound 37.*

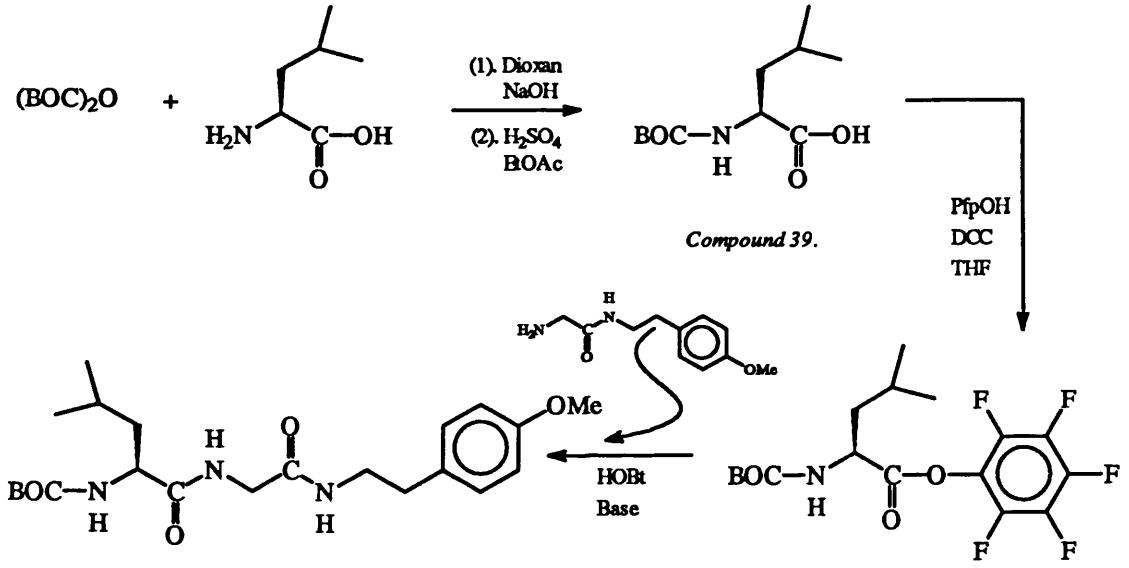
Compound 38. *Scheme 3.18.*

3.5.1.3. The Tetra peptide Compounds. (GlyPheLeuGly).

The iterative addition of leucine to glycine N-(2-(4-methoxyphenyl)ethyl)amide (*Compound 37*) would provide a useful intermediate to the tetrapeptide formation. The direct coupling of BOC leucine to the primary amine using a DCC coupling regimen was dismissed following the disappointing yields previously obtained with this method. However, α-amino protection of leucine with BOC (249) in good yield enabled the amino acid to be activated, (*Scheme 3.19*). Pentafluorophenol was the reagent of choice as it is especially reactive and does not interfere with the chiral integrity of susceptible compounds. The methodology was the same as for *Compound 28* and the ester was afforded in good yield. The melting point of *Compound 40* was in agreement with the

literature value (332) and a mass spectral peak for the M + H ion at *m/z* 398 substantiated the identity of the product.

An attempt was made to treat glycine N-(2-(4-methoxyphenyl)ethyl)amide (*Compound 37*) with BOCLeuOPfp in the presence of diisopropylethylamine (hindered non-nucleophilic base) and HOBt, (*Scheme 3.19*). A crude product which contained some active ester starting material was washed with aqueous base, with no effect. Direct application of the crude reaction mixture to a chromatography column and subsequent elution permitted the isolation of a single product in moderate yield. This material was identified as *Compound 41* by ^{1}H NMR, mass spectroscopy and elemental analysis.

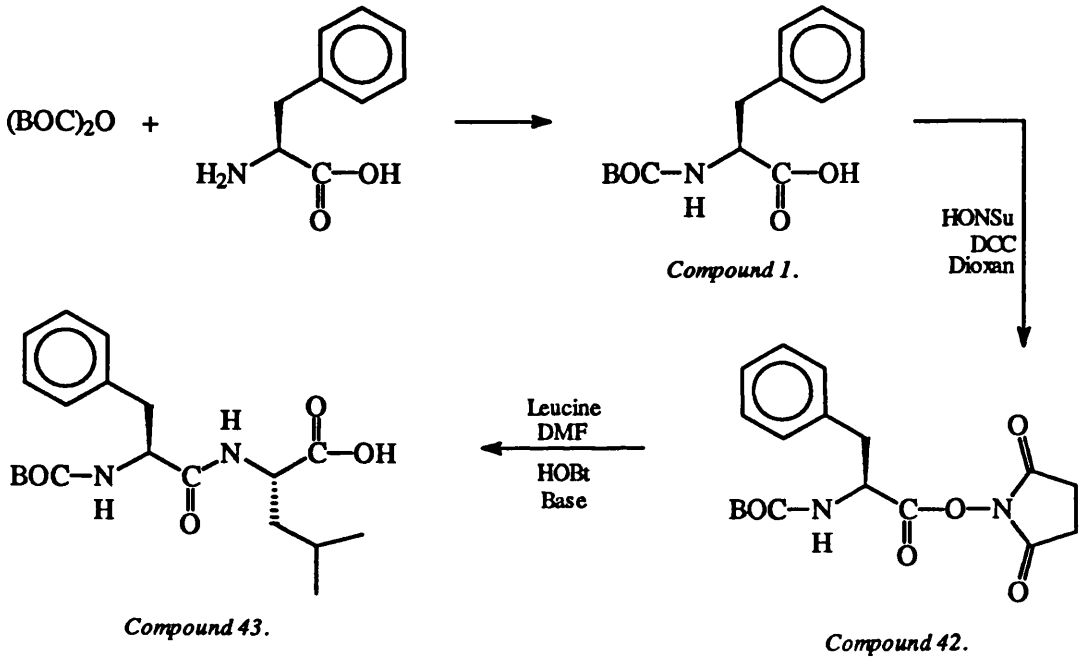


Scheme 3.19.

If the free amine of BOCLeuGly N-(2-(4-methoxyphenyl)ethyl)amide (*Compound 41*), is to undergo iterative amino acid elongation to form a tripeptide compound, then the BOC protecting group must be removed. In addition, the amino acid to be introduced into

the chain, *i.e.* phenylalanine must be amino protected and activated in order to react with the leucyl amino group. Finally, the tripeptide must be deprotected in readiness to accept the next amino acid. A more convergent strategy to lengthen the peptide chain involved the reaction of *Compound 37* with the activated dipeptide, BOCPheLeuOPfp.

BOC phenylalanine, (*Compound 1*) was synthesised in good yield following a similar methodology to that for BOCGly and BOCLeu, (*Scheme 3.20*).

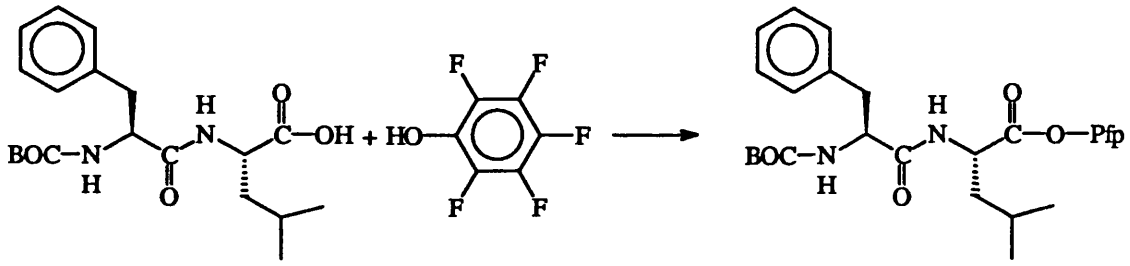


Scheme 3.20.

The BOCPhe was converted to the active ester by treatment with N-hydroxysuccinimide (HONSu) using dicyclohexylcarbodiimide. Following the removal of the DCU from the dioxan, the evaporation residue from the filtrate was triturated from ether to give the known compound BOCPheOSu (*Compound 42*) (250). This material was identified by spectroscopic, melting point and elemental analysis.

The dipeptide was conveniently prepared by the reaction of the N-hydroxy-succinimide derivative and leucine in a mixture of organic solvent and aqueous base according to the method of Bower *et al* (251). The product was afforded in good yield. The disappearance of the four proton singlets at δ 2.79 in the ^{1}H NMR spectrum implied that the active ester had been removed, and further peaks integrated accurately to corroborate the identity of *Compound 43*.

The acyl dipeptide ester (*Compound 44*) was obtained in good yield from a DCC coupling reaction in a similar manner to the formation of BOCLeuOPfp (*Compound 40*), (*Scheme 3.21*).



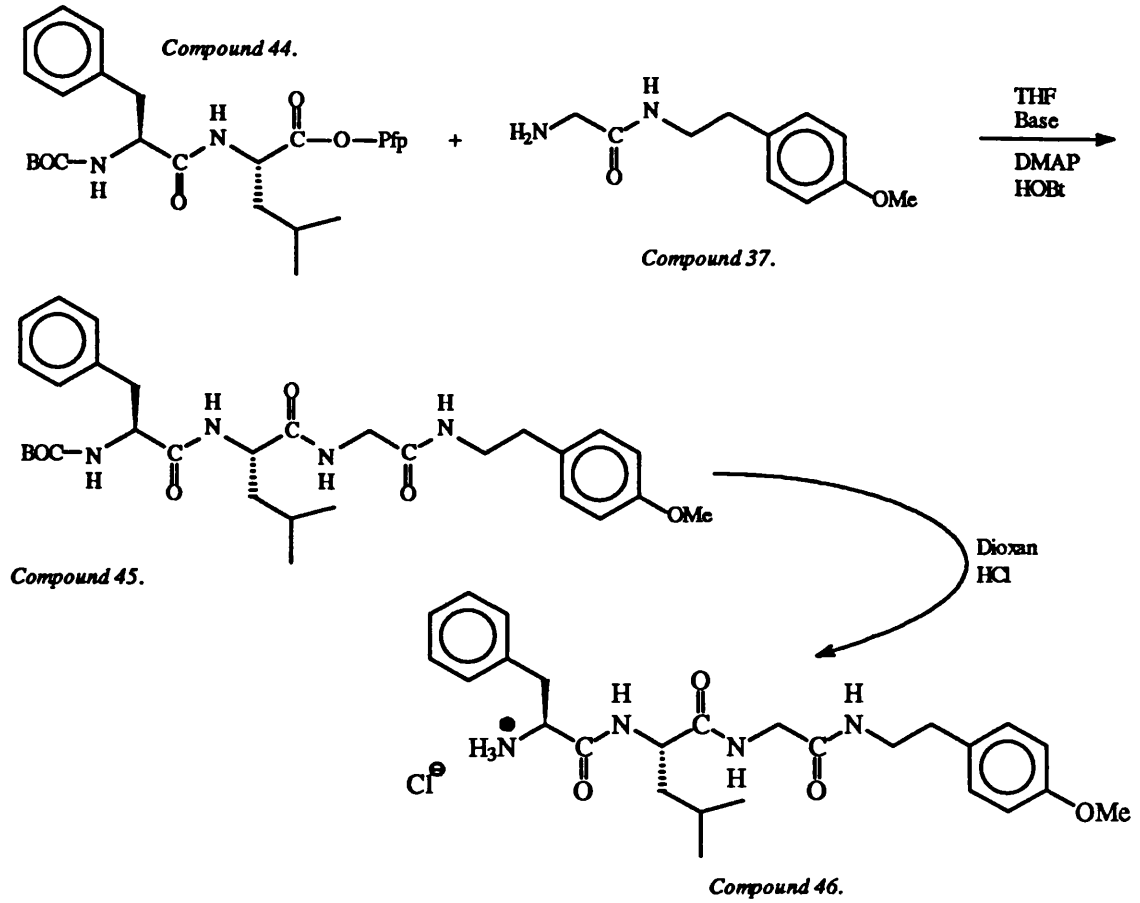
Compound 44.

Scheme 3.21.

The identity of *Compound 44* was confirmed by elemental analysis mass spectroscopy by the observation of the M + H ion at *m/z* 545. The electron-withdrawing effect of the Pfp group on the leucyl α-proton could not be compared accurately with BOCPheLeu since the spectra were generated using different deuterated solvents. However, the presence of the aromatic phenyl cluster at δ 7.30, *t*-butyl at δ 1.41, and quartet of the α-Leu proton at δ 4.40 corroborated the identity of the product.

The choice of active ester was partly dictated by the reactive nature of the pentafluorophenyl esters and partly by issues of racemisation. There is a risk of racemisation when amino acids with chiral centres, *i.e.* Phe and Leu, are coupled together, directly (DCC) or indirectly (active ester). This risk is increased when active esters are coupled, in the prescence of base, from susceptible carboxy components. However, the Pfp esters are regarded as being less susceptible to racemisation and the formation of stereoisomeric compounds is therefore avoided.

An important step in the synthesis of the final tetrapeptide compound was the coupling of glycine N-(2-(4-methoxyphenyl)ethyl)amide (*Compound 37*) to BOCPheLeuOPfp (*Compound 44*) to form the tripeptide conjugate BOCPheLeuGly N-(2-(4-methoxy-phenyl)ethyl)amide, (*Compound 45*). By slightly modifying a standard procedure to include a small amount of DMAP (a nucleophilic catalyst), *Compound 45* was afforded in moderate yield, (*Scheme 3.22*). The ^{1}H NMR spectrum contained no duplicate signals for the product, indicating the presence of only one diastereoisomer and it was concluded that racemisation had been avoided. All the signals were assigned, and the product melted sharply over a one degree range.

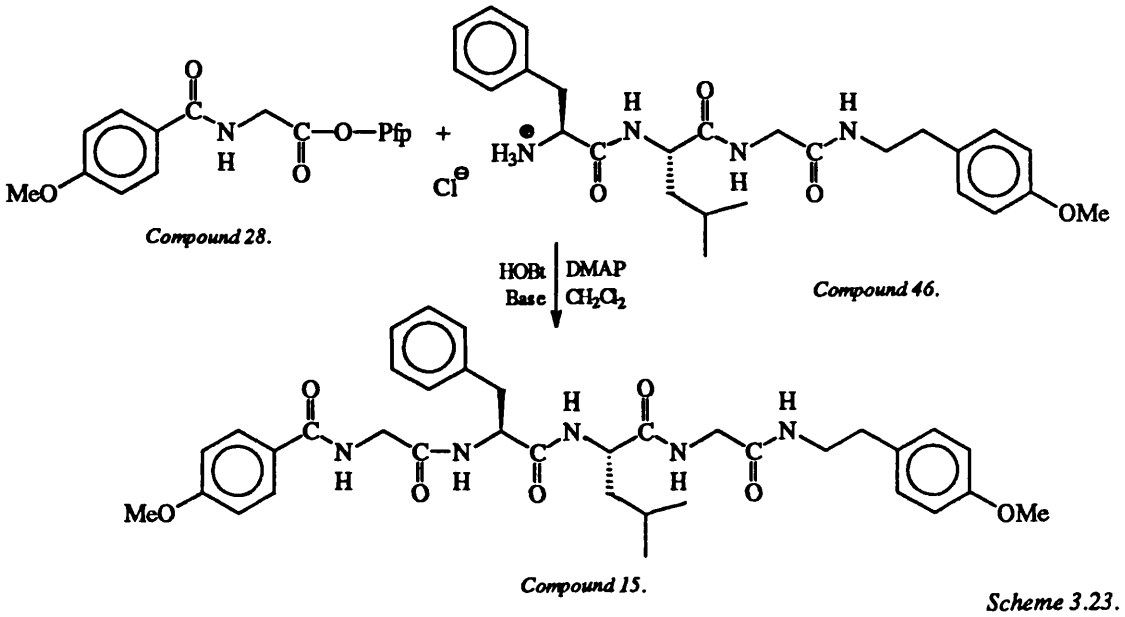


Scheme 3.22.

The hydrochloride salt of deprotected *Compound 46* (PheLeuGly N-(2-(4-(methoxyphenyl)ethyl)amide hydrochloride) was isolated in moderate yield by removing the BOC protecting group with hydrogen chloride. This salt was treated with N-(4-methoxybenzoyl)glycineOPfp (*Compound 28*) in the presence of excess triethylamine base. This is in contrast to the previous experiments where the free amine was allowed to react with an activated ester. Both synthetic strategies have been shown to be successful. However, by shortening the synthetic route, the product yield was conserved.

Since N-(4-methoxybenzoyl)glycineOPfp has been shown to react well with a primary amine, the synthesis of the target tetrapeptide compound was investigated. The hydrochloride salt of PheLeuGly N-(2-(4-methoxyphenyl)ethyl)amide (*Compound 46*) was

added dropwise to a dilute mixture of 4-methoxybenzoylGlyOPfp / HOBt / DMAP and an excess of base which exposed the free amine *in situ*, (*Scheme 3.23*). Removal of the organic solvent gave the crude product as a multi-component mixture, but direct application to a chromatography column and subsequent elution permitted the isolation of a single product in satisfactory yield. This material was identified by mass spectroscopy, with observation of the M + H ion at *m/z* 660 and the M - H ion at 658 for FAB [+] and FAB [-] respectively. This characterisation was confirmed by ^{1}H NMR, (*Compound 15*).



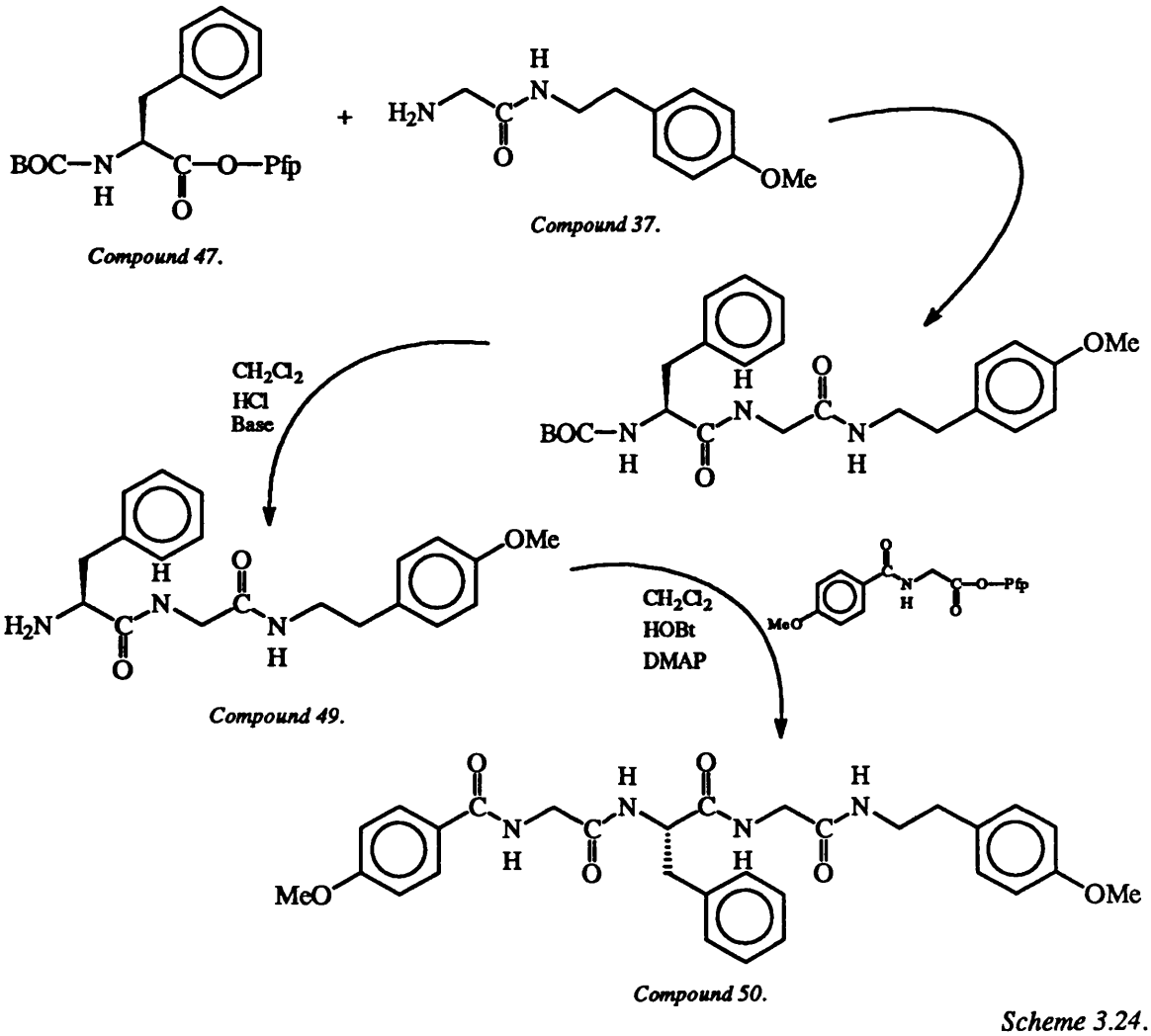
Compound 28. *Compound 46.* *Compound 15.*

Scheme 3.23.

3.5.1.4. The Tri Peptide Compounds. (GlyPheGly).

In an attempt to synthesise a tripeptide model compound, BOCPheOPfp (*Compound 47*) was allowed to react with glycine N-(2-(4-methoxyphenyl)ethyl)amide, (*Compound 37*). The protected dipeptide was converted to the N-(phenylalanyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide hydrochloride (*Compound 49*) and treated in a catalysed reaction with N-(4-methoxybenzoyl)glycineOPfp in an excess of base.

Conventional work-up gave a crude product that was separated by column chromatography. Subsequent elution permitted the isolation of a product, the bis(4-methoxyaryl) substituted GlyPheGly (*Compound 50*) in fair yield, (*Scheme 3.24*).



Compound 47.

Compound 37.

Compound 49.

Compound 50.

Scheme 3.24.

3.5.2. Benzyloxy work

A considerable amount of time was spent in optimising the experimental conditions that led to the formation of the bis(methoxyaryl)tetrapeptide compound, (*Compound 15*). Unfortunately, the progress of this work was abruptly terminated when deprotection of

Compound 15 proved problematic (*Section 2.4.3.*). An alternative, more successful, approach involved substituting benzyloxyphenyl side groups onto the tetrapeptide chain, (*Figure 3.17*). As a close relationship exists between the previous target compounds and those discussed presently, many of the same synthetic approaches have been adopted.

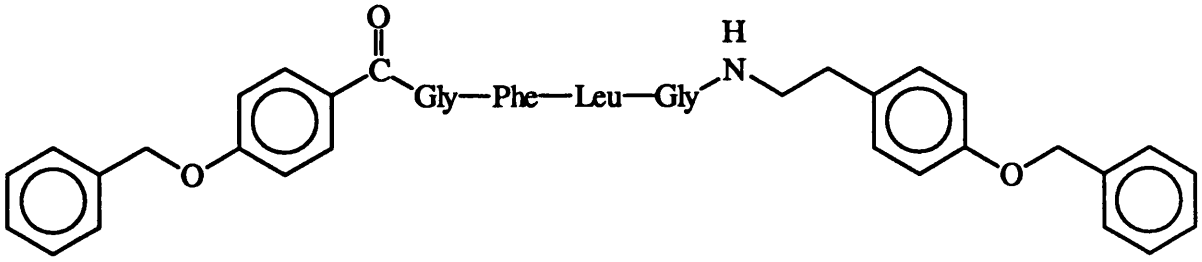
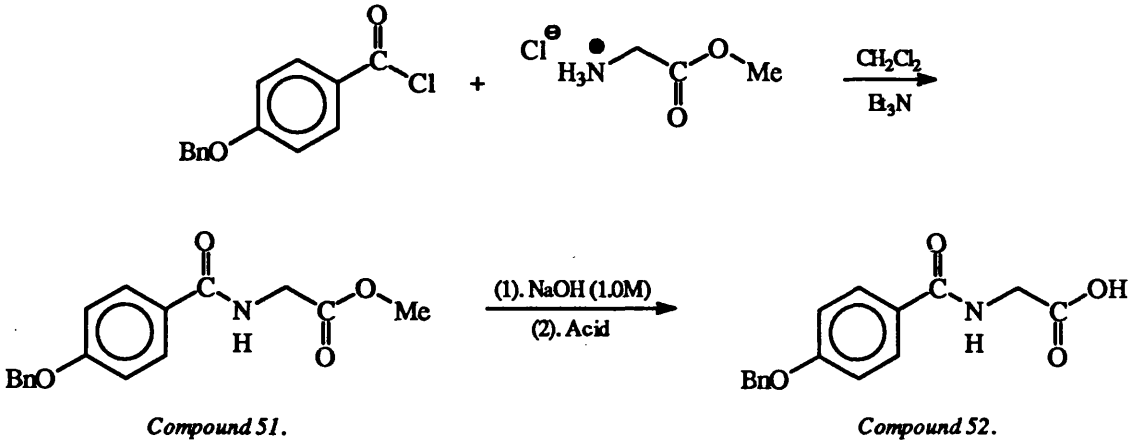


Fig. 3.17.

3.5.2.1. The Di Peptides. (GlyGly).

Since the glycine esters have been shown to be more amenable than glycine for reaction with an acid chloride, synthesis of N-(4-(phenylmethoxy)benzoyl)glycine methyl ester was investigated. Glycine methyl ester hydrochloride was suspended in dichloromethane and converted to the free amine by the addition of excess triethylamine. The aryl acid chloride was carefully added to this buffered solution and allowed to react, before a conventional work-up afforded a product which was recrystallised in good yield, (*Scheme 3.25*). This material was identified by ^{1}H NMR spectroscopy. Study of the NMR spectral data showed a singlet at δ 3.78, assigned to the methyl ester and an aromatic cluster between δ 6.98 and δ 7.77 which corroborated the identity of *Compound 51.*



Compound 51. *Compound 52.*

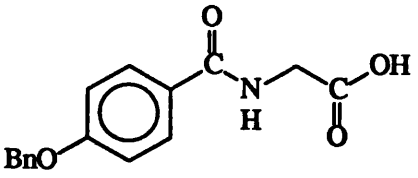
Scheme 3.25.

Removal of the methyl ester group by base hydrolysis from *Compound 51* formed an α-carboxylic acid component for activation with a suitable leaving group. Removal of the solvent afforded a material in good yield that was analysed by standard techniques. Mass spectroscopy confirmed the presence of an M + H ion at *m/z* 286 and an M - H ion at 284 for FAB [+] and FAB [-] respectively. This characterisation was corroborated by ^{1}H NMR which showed the replacement of the methyl singlet peak at δ 3.78 with a broad peak at δ 12.60, which was assigned to the COOH, (*Compound 52*).

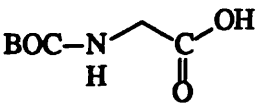
The activated ester was prepared using pentafluorophenol in an analogous experiment to that for *Compound 28*. A product was separated by column chromatography in good yield and was identified as *Compound 53*, by elemental analysis, mass spectroscopy and proton NMR spectroscopy, (*Scheme 3.26*).

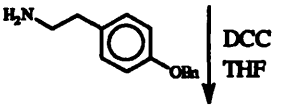
By slightly modifying a standard procedure, the active pentafluorophenyl ester of *Compound* 53 was successfully treated with glycine N-(2-(4-phenylmethoxy)phenyl)ethyl amide hydrochloride (*Compound 55*). The latter compound was prepared in good yield *via* the DCC coupling of BOCGly and N-(2-(4-phenylmethoxy)phenyl)ethylamine

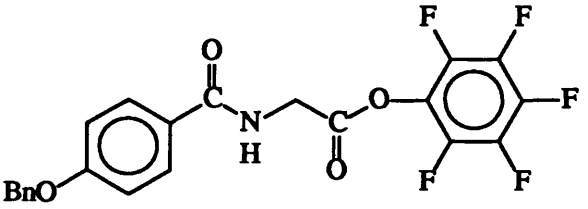
(*Compound 32*). Subsequent α-amino deprotection of *Compound 54* in dioxan with hydrogen chloride afforded the amine salt in good yield, a small portion of which was treated with aqueous base to give the free amine. The chloride salt of *Compound 55* was dissolved in dry dichloromethane with an excess of N,N-diisopropylethylamine (a hindered base) to facilitate the formation of the free amine *in situ*, (*Scheme 3.26.*). N-(2-(4-phenylmethoxy)benzoyl)glycine pentafluorophenol ester was added dropwise and the mixture stirred for 6 hours. The organic solvent in the filtrate was evaporated to afford a multi-component white powder which was separated to give a single product in poor yield following column chromatography. This material was identified as the dipeptide (*Compound 21*) by ^{1}H NMR and COSY. This characterisation was confirmed by observation of the M + H ion at *m/z* 552 and the M - H ion at *m/z* 550 in the FAB [+] and FAB [-] mass spectrum, respectively, (*Scheme 3.26*).



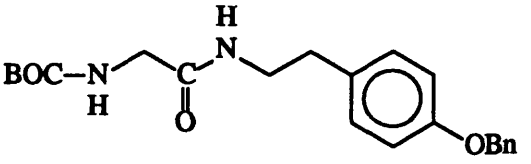
Compound 52.



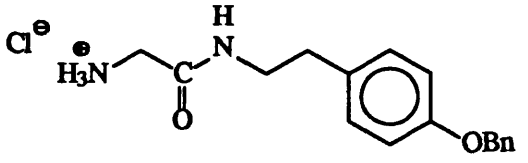




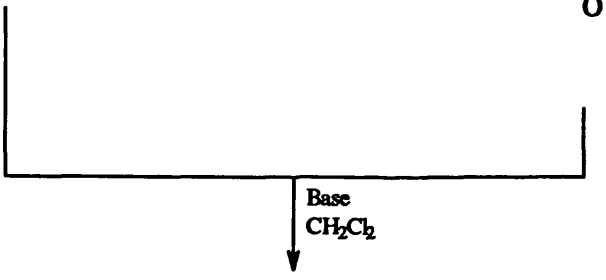
Compound 53.

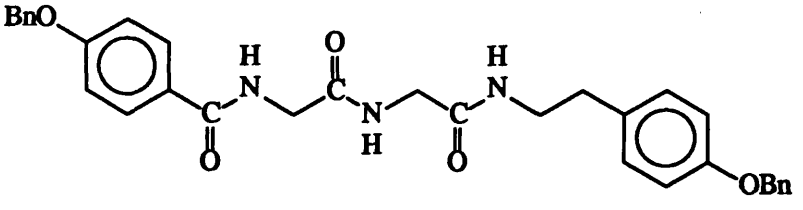


Compound 54.



Compound 55.





Compound 21.

Scheme 3.26.

3.5.2.2. The tripeptide PheLeuGly.

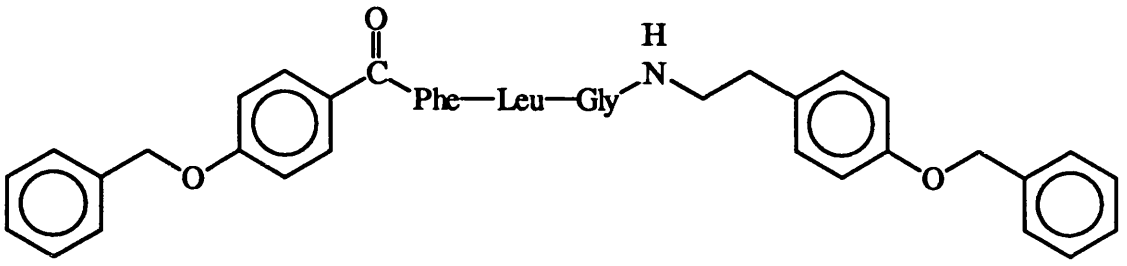


Fig. 3.18.

The strategic approach adopted for the preparation of the tripeptide analogue (*Figure 3.18*) was based on previous work (Section 3.5.1.). Since BOCPheLeuOPfp (*Compound 44*) had been successfully coupled to glycine N-(2-(4-methoxyphenyl)ethyl)-amine, the synthesis of N-(N-(N-(BOC)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide was investigated, (*Figure 3.19*).

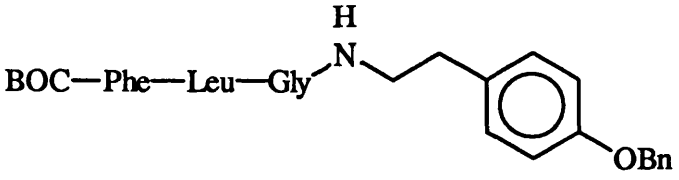
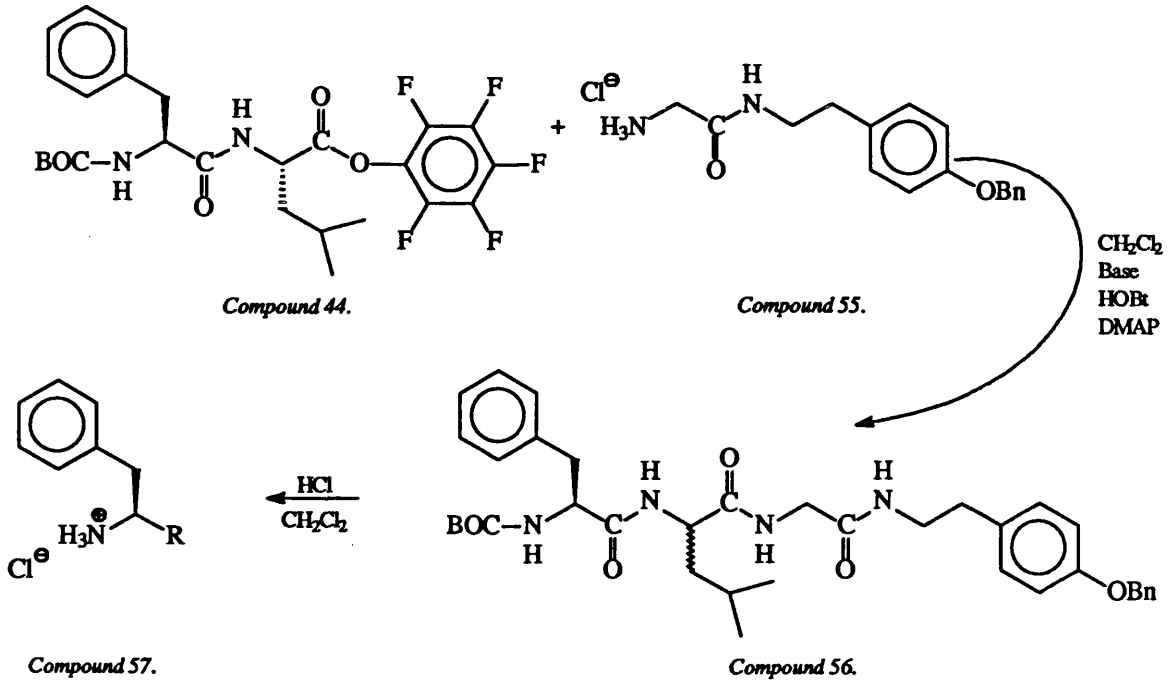


Fig. 3.19.

The hydrochloride salt of *Compound 55* [glycine N-(2-(4-(phenylmethoxy)phenyl)-ethyl)amide] was converted to the free amine using N,N-diisopropylethylamine in an organic solvent, (*Scheme 3.27*). The pentafluorophenyl ester (*Compound 44*) was treated with the free amine *in situ* in a reaction catalysed by DMAP and HOBt. Following a brief wash with aqueous acid, the organic solvent was evaporated to give a multi-component oil. Application of the crude material to a chromatography column and subsequent elution permitted the isolation of a single product, as a solid, in moderate yield, (*Compound 56*). Characterisation of this material with ^{1}H NMR identified the desired compound, together

with an excessive number of signals. Further extrapolation of the proton spectrum suggested that the material existed as a mixture of diastereomeric compounds.



Compound 44. *Compound 55.* *Compound 57.* *Compound 56.*

Scheme 3.27.

Surprisingly, the synthesis of BOCPheLeuGlyN-(2-(4-(phenylmethoxy)phenyl)-ethyl)amide (*Compound 56*) resulted in a mixture of diastereoisomers, which was unexpected for a number of reasons. Firstly, ^{1}H NMR data from the optically-active starting materials (BOCPheLeu (*Compound 43*) and BOCPheLeuOPfp (*Compound 44*)) showed no loss of chiral integrity (*Figure 3.20 (a) and (b)*). Secondly, the synthesis of an analogous compound, BOCPheLeuGly N-(2-(4-methoxyphenyl)ethyl)amide (*Compound 45)* using a similar experimental protocol had shown no signs of racemisation (*Figure 3.20 (c)*).

Proton spectroscopy was used to compare the diastereomeric mixtures using the shift values of four leucine γ-methyl doublets, which could be resolved more easily from

the "busy" spectra, (*Figure 3.20 and 3.21*). Isomerically pure precursors containing leucine enabled the two doublets at δ 0.87 and δ 0.89 to be identified as the L-isomer, whereas the more shielded doublets at δ 0.73 and δ 0.80 were assigned to the D-isomer (*Figure 3.20 (d)*). Comparison of the integrals for the leucine γ-methyl L and D doublets showed a 2 : 1 ratio of L-isomer to D-isomer. An attempt to separate this mixture by column chromatography was not successful and provided a product with the same ratio of diastereoisomers. Characterisation of *Compound 56* was corroborated by observation of the M + H ion at *m/z* 645 for FAB [+] and the M - H ion at *m/z* 643 for FAB [-] in the mass spectra.

The formation of diastereoisomers, elucidated from the NMR data suggested that either the phenylalanine or leucine or both had undergone racemisation during the coupling reaction. If only the leucine chiral centre had been effected then the material would exist as a mixture of L, L or L and D diastereoisomers. If, on the other hand, both asymmetric centres had been involved then the compound would exist as a mixture of both diastereoisomers and their enantiomers, *i.e.*, L L; L D; D D; D L. The enantiomers to the diastereoisomers are invisible to NMR spectroscopy since they are identical in most physical properties. However, they do differ in the way they interact with plane polarised light. In order to determine if one or both of the chiral centres of *Compound 56* had racemised the optical rotation of the material was measured. A specific rotation measurement of $[\alpha]^{23}_{589}$ +24.6° suggested that the compound did not exist as a racemic mixture, as this has no optical activity. It was concluded therefore that only the leucine had racemised and the material contained both L-Phe L-Leu and L-Phe D-Leu.

The integrity of the chiral precursors to *Compound 56* were also investigated and found to be $[\alpha]^{23}_{589}$ + 10.4° and $[\alpha]^{23}_{589}$ -14.4° and for BOCPhe and BOCPheLeu, respectively.

The BOC group of *Compound 56* was cleaved using hydrogen chloride in dichloromethane. Evaporation of the solvent provided a white solid in good yield. This material was identified as a mixture of diastereoisomers of the hydrochloride salt of the tripeptide, (*Compound 57*). As expected, the broad singlet associated with BOC *circa* δ 1.3 was absent from the ^{1}H NMR spectrum of *Compound 57*, but a new broad singlet which integrated correctly for the phenyl $^+NH_3$ was observed at δ 5.58. Both diastereoisomers of *Compound 57* were assigned within the spectrum, but a comparison of the integrals of the leucyl γ-bis(methyl) groups showed an approximate 1:1 ratio of diastereoisomers.

Figure 3.20. A Comparison of ^1H NMR Leucine-γ-Methyl Signals for Chirally Active Intermediate Compounds.

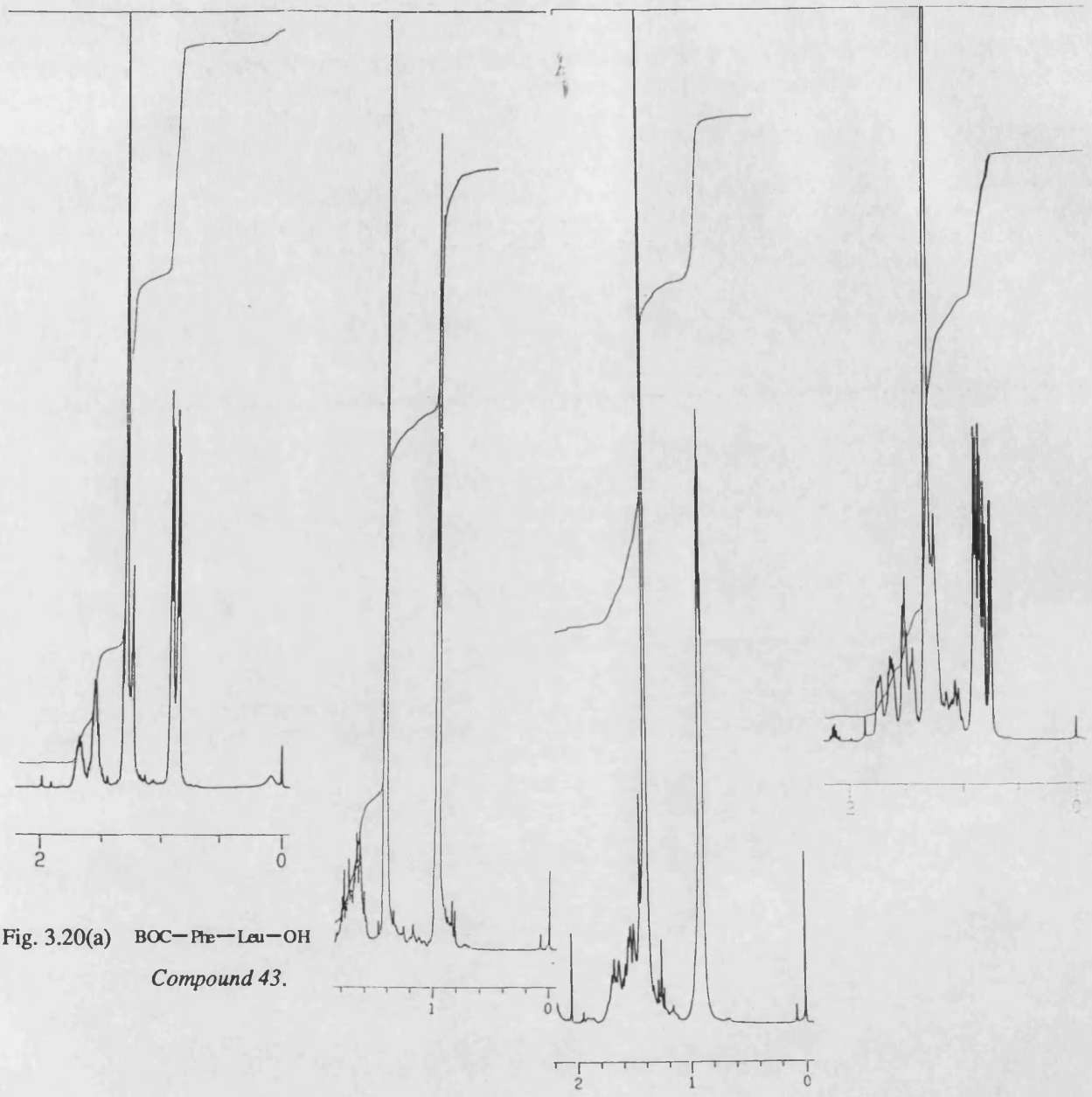
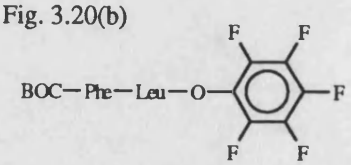


Fig. 3.20(a) BOC—Phe—Leu—OH

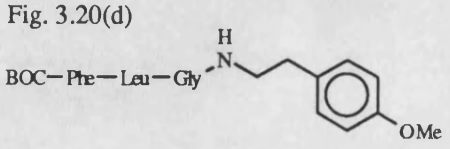
Compound 43.

Fig. 3.20(b)



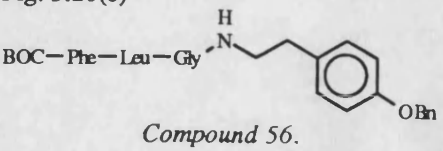
Compound 44.

Fig. 3.20(d)



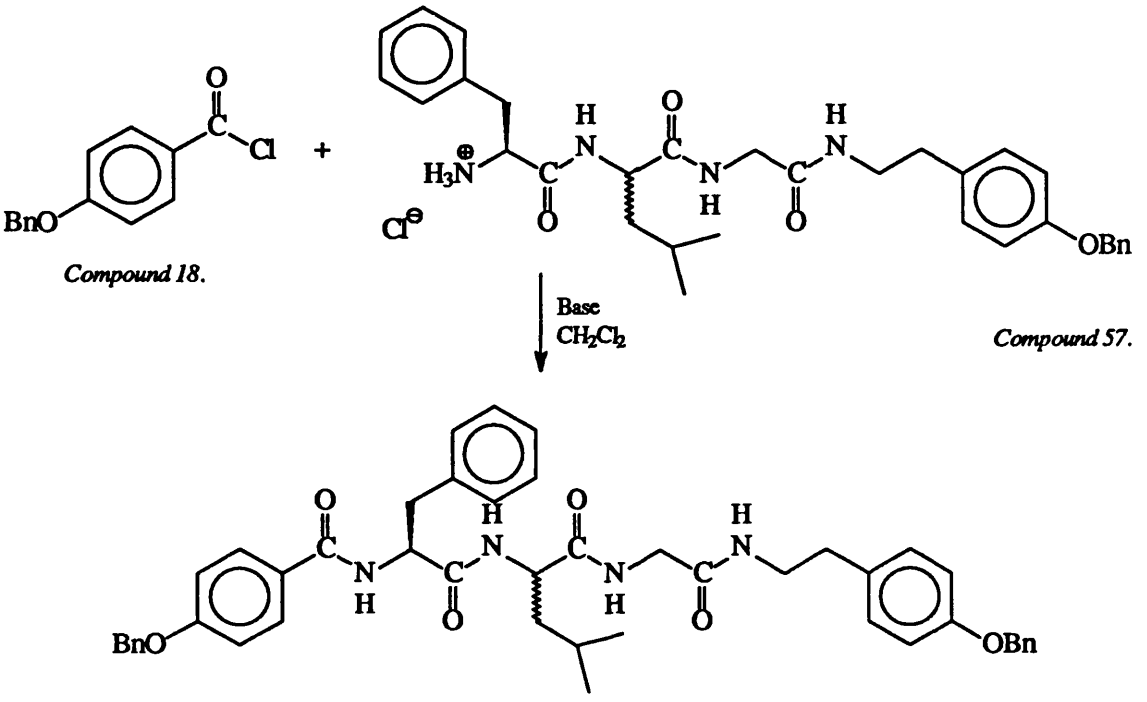
Compound 45.

Fig. 3.20(c)



Compound 56.

The possibility of preparing further conjugates with a peptidic hydrochloride salt has been used successfully in previous experiments. An attempt was therefore made to treat 4-(phenylmethoxy)benzoyl chloride (*Compound* 18) in dichloromethane with an excess of base and an equimolar amount of the tripeptide hydrochloride salt (*Compound 57*), (*Scheme 3.28*).



Compound 18. *Compound 57.*

Compound 22. *Scheme 3.28.*

Evaporation of the solvent afforded a multi-component residue which was applied directly to a chromatography column. Spectroscopy of an isolated product provided further evidence of the diastereomeric nature of the product (*Figure 3.21 (b)*). Correlation spectroscopy (COSY) together with ^1H NMR enabled each signal in the spectrum to be assigned to the respective isomer (*See spectrum 2, Appendix 2*). The identity of the product (*Compound 22*) was corroborated by accurate mass spectroscopy with the observation of an ion at *m/z* 756.3892 (calculated 756.3887).

Figure 3.21. A Comparison of ^1H NMR Leucine-γ-Methyl Signals for Chirally Active Products.

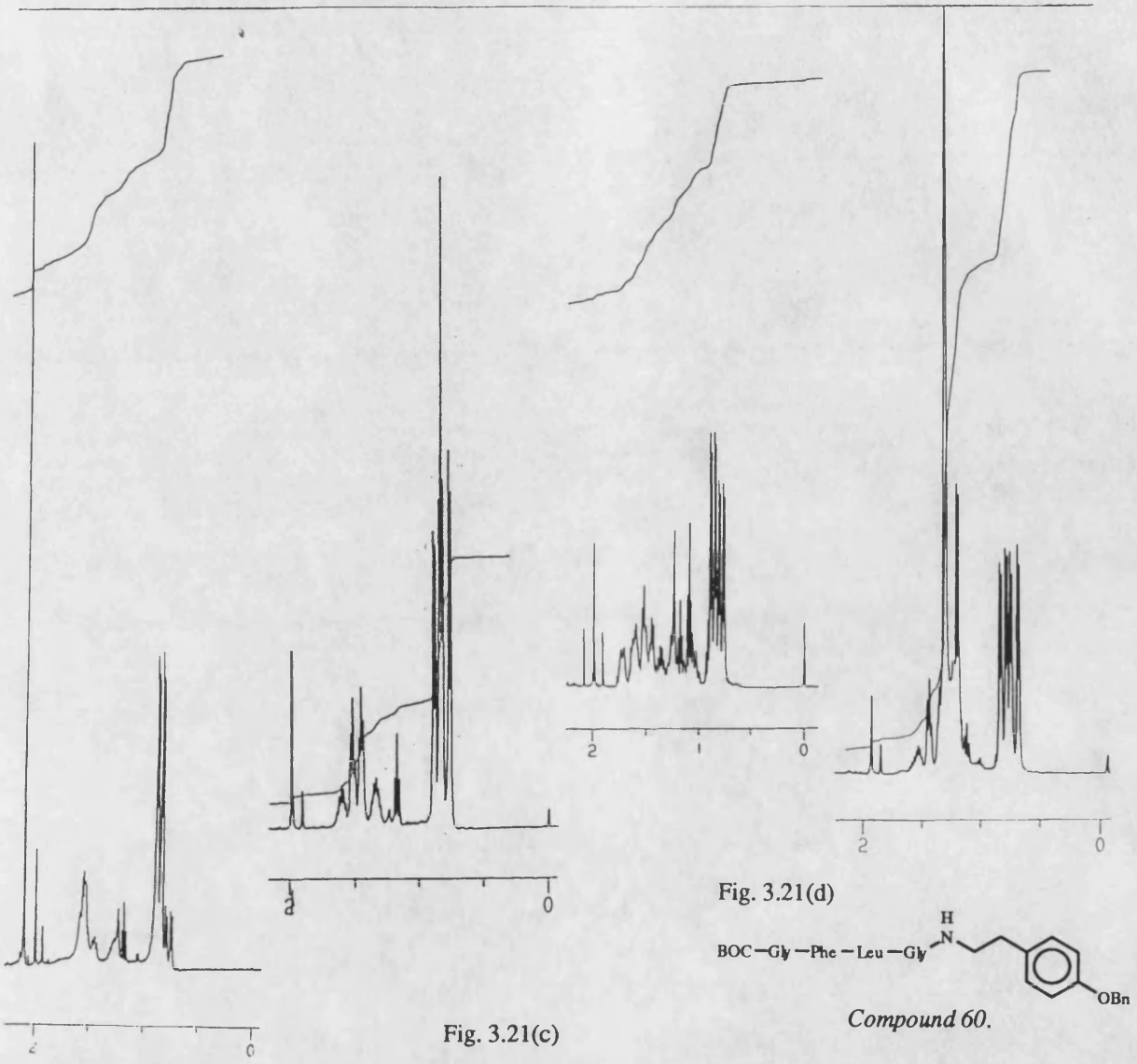
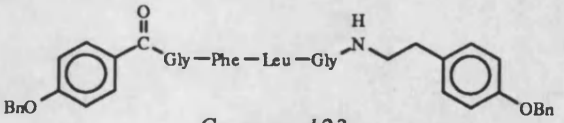


Fig. 3.21(d)

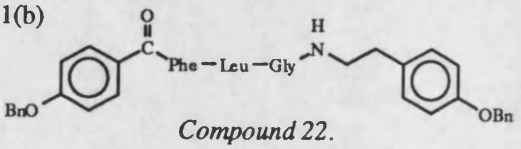
Compound 60.

Fig. 3.21(c)



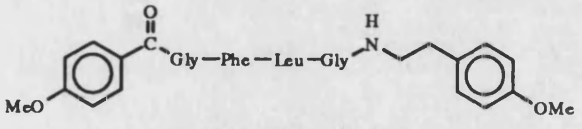
Compound 23.

Fig. 3.21(b)



Compound 22.

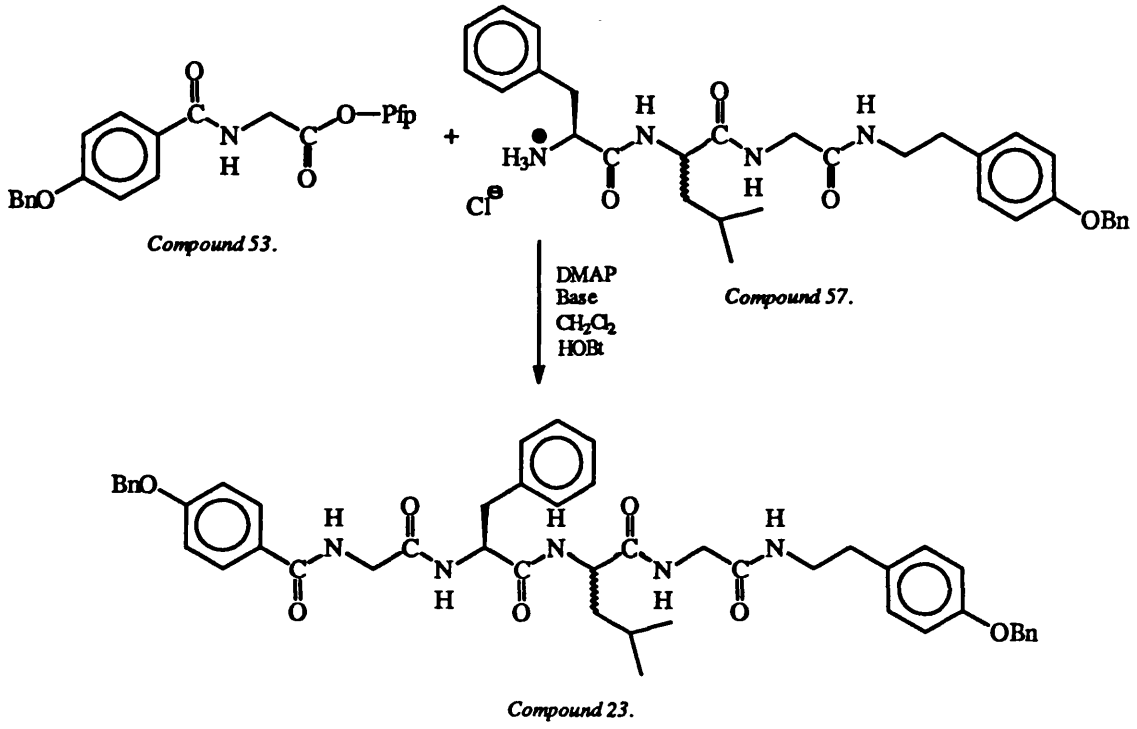
Fig. 3.21(a)



Compound 15.

3.5.2.3. The tetrapeptide - GlyPheLeuGly

In analogy to the known reaction of aryl carboxypeptideOPfp and peptidic hydrochloride salt, the arylGlyOPfp (*Compound* 53) was allowed to react with the hydrochloride salt of the tripeptide (*Compound 57*), (*Scheme 3.29*).



Scheme 3.29.

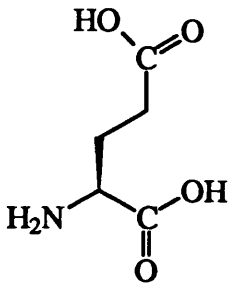
The hydrochloride salt (*Compound 57*) was suspended in dichloromethane and converted to the free amine by the addition of excess N,N-diisopropylethylamine (base). A catalytic amount of DMAP and HOBt was added to the solution before an equimolar amount of N-(2-(4-(phenylmethoxy)benzoyl)glycineOPfp was allowed to react *in situ* with the mixture. On evaporation of the solvent, the resulting gum was applied directly to a chromatography column and a single product was eluted in good yield. The ratio of diastereoisomers was not changed during chromatographic separation according to ^{1}H NMR (*Figure 3.21 (c)*),

however, the spectra corroborated the proposed identity of the product as that of the target compound, *Compound* 23.

3.5.3. Attachment of ligands.

According to Duncan *et al* (252) attachment of a drug to a macromolecular carrier can be used to manipulate biodistribution, permeability through biological barriers and pharmacokinetics. However, the proposed PEG-peptide polymers under investigation, have been designed in a linear fashion with amino acids selected for their lability to enzymatic degradation. The lack of peptide functional groups severely limits not only the drug" loading" of such conjugates, but also the design options for PEG-peptide based drug carrier systems. To circumvent this limitation, we attempted to prepare copolymers of PEG and peptide containing reactive pendant groups along the polymer backbone.

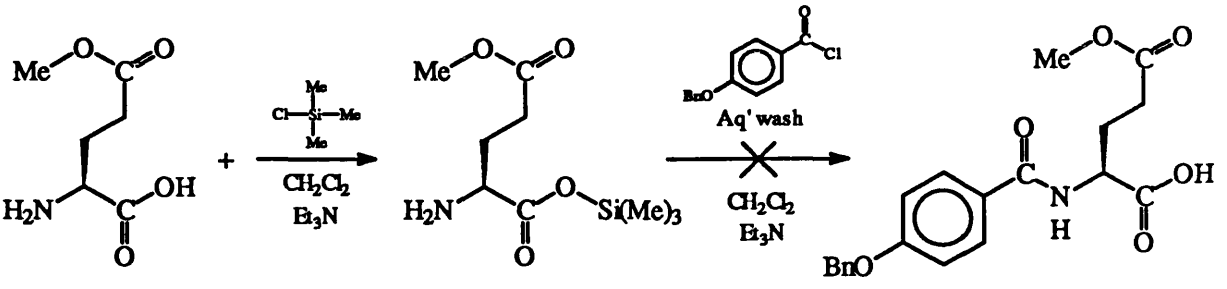
Controlled peptide bond formation with glutamic acid (*Figure 3.22*) involving the α-carboxy group absolutely requires obstruction of the γ-carboxy group. In addition the protecting group may need to be orthogonal to the α-amino and α-carboxy protecting groups.



Glutamic acid.
Fig. 3.22.

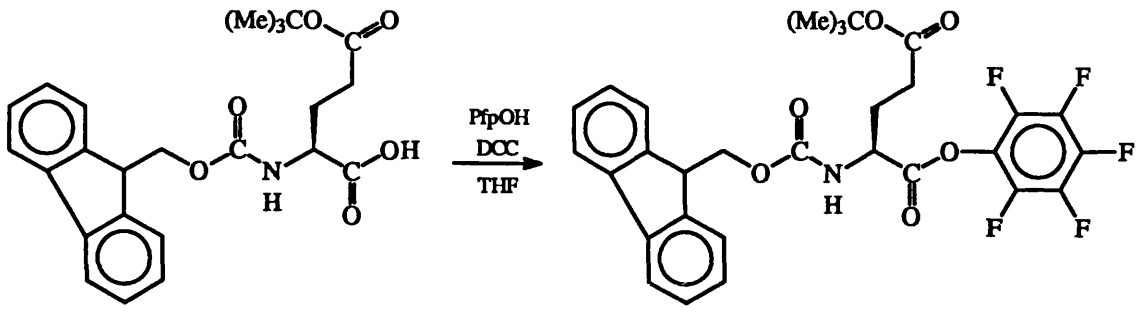
An initial attempt to incorporate glutamic acid into the tetrapeptide chain involved the reaction of 4-(phenylmethoxy)benzoyl chloride and a α,γ-carboxy protected glutamic

acid. A method adapted from Schwarz *et al* (252a) involved the introduction of trimethylsiloxy onto the α-carboxy compartment of a γ-methyl protected glutamic acid, (*Scheme 3.30*). However, treatment of the carboxy protected compound with the acid chloride gave no material which could be identified as the required product.



Scheme 3.30.

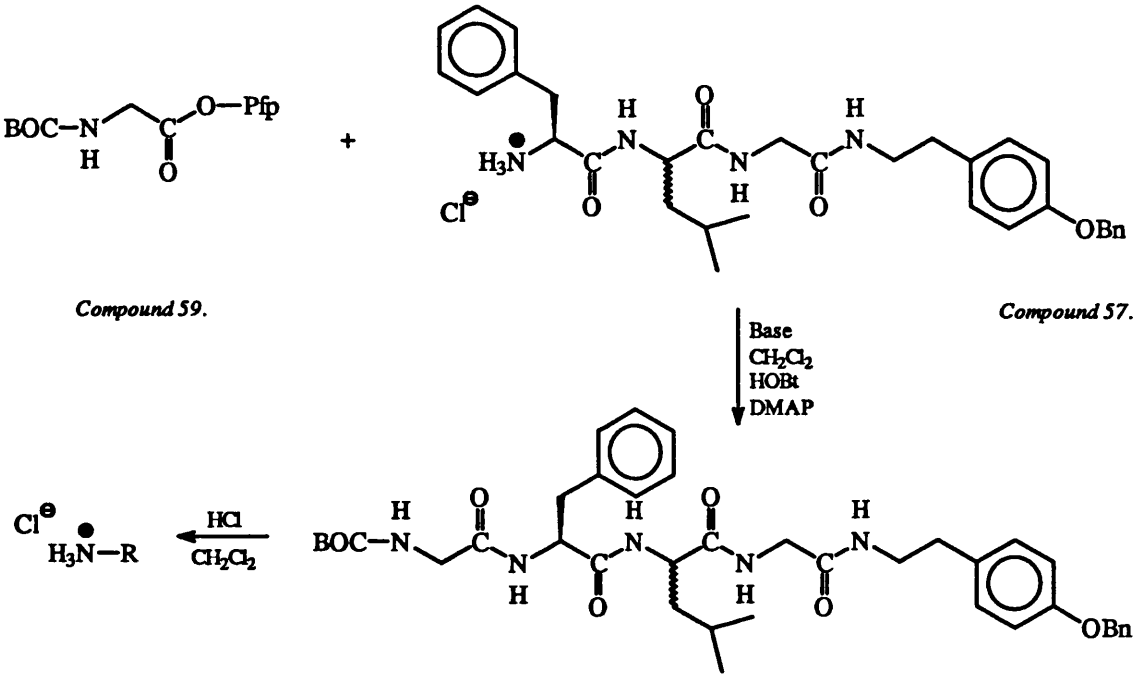
Since FMOCglutamic acid-γ-*t*-(butyl) ester provides a suitable orthogonal protecting group to the benzyloxy tetrapeptide chain, (*Compound 23*) the synthesis of an α-amino FMOC pentapeptide compound was investigated. The α-amino, γ-carboxy protected glutamic acid was converted to an active ester using pentafluorophenol in a DCC coupling reaction, (*Scheme 3.31*). Conventional work-up afforded a single product in good yield which was characterised *inter alia* by ^{19}F NMR. Comparison of the shift values for the ortho, para and meta fluorine signals at -152.69, -157.63 and -162.21 respectively showed a considerable de-shielding effect compared to the pentafluorophenol. A trace amount of pentafluorophenol was detected in the spectrum which was not removed from *Compound 58*.



Compound 58.

Scheme 3.31.

To provide the appropriately derivatised tetrapeptide the activated ester of glycine (*Compound 59*) was synthesised by treating BOCglycine (249) with pentafluorophenol in the presence of DCC. By slightly modifying a previously discussed procedure, the reaction between *Compound* 59 and the tripeptide (*Compound* 57) was investigated. A solution of the hydrochloride salt of *Compound* 57 was treated with an excess of base in dichloromethane. The tripeptide, as a free base, was allowed to react with BOCGlyOPfp *in situ* in the presence of HOBt and DMAP. A single product was eluted from a chromatography column after a conventional work-up, and identified as the desired tetrapeptide (*Compound* 60) in good yield, (*Scheme 3.32*).

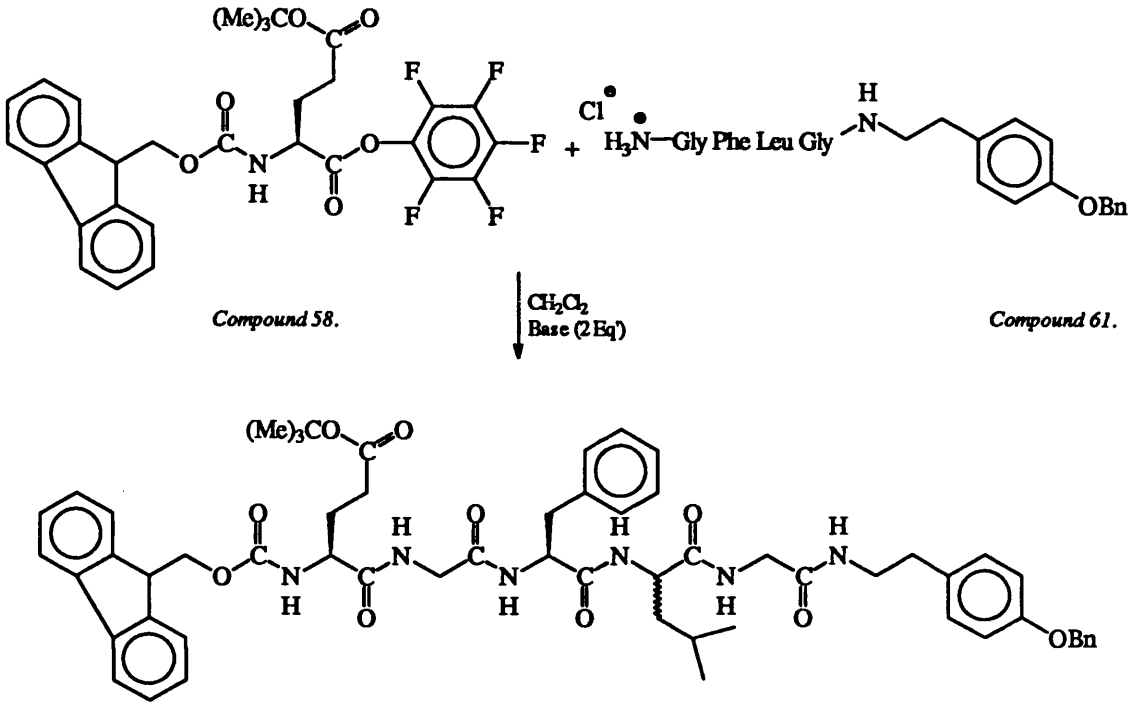


Compound 59. *Compound 57.*

Compound 61. *Compound 60.*

Scheme 3.32.

Since FMOCGlu(γ-*t*Bu)OPfp (*Compound 58*) is susceptible to nucleophilic attack, the hydrochloride salt of *Compound 61* was converted to the free amine. *Compound 58* and *Compound 61* were suspended in dry dichloromethane and care was taken to add an exact equimolar amount of N,N-(diisopropyl)ethylamine to the mixture. The FMOC protecting group is cleaved swiftly under alkaline conditions and the base was therefore added dropwise over one hour. Removal of the solvent by evaporation gave a crude product but direct application to a chromatography column and subsequent elution permitted the isolation of a single product in good yield, (*Scheme 3.33*).



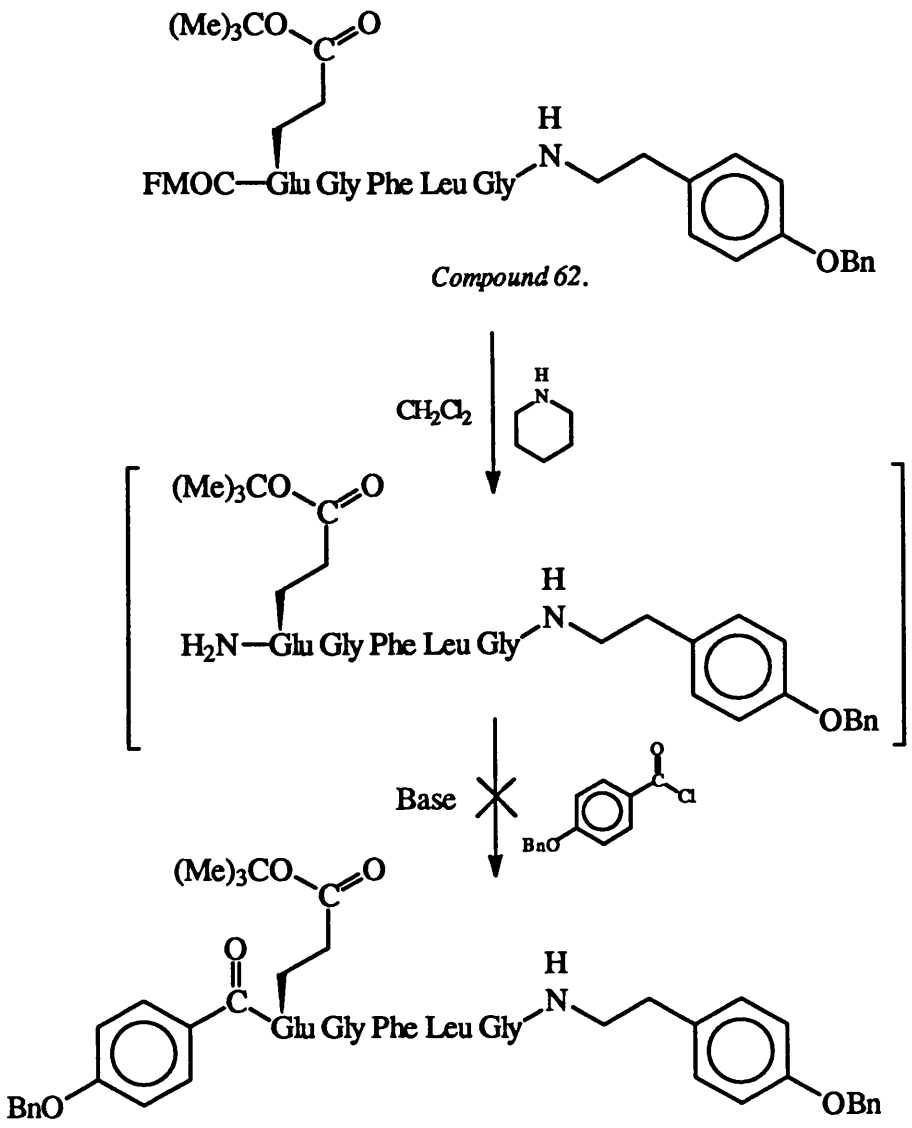
Compound 58. *Compound 61.*

Compound 62.

Scheme 3.33.

This material was characterised by accurate mass spectroscopy with the observation of an M + H ion at *m/z* 1008.5023 (requires 1008.4997). The identity of *Compound 62* was further corroborated by ^{1}H NMR and COSY analysis, (*see Spectrum 3, Appendix 3*).

In order to synthesise the target pentapeptide compound an attempt was made to couple 4-(phenylmethoxy)benzoyl chloride, (*Compound 18*) *in situ* with freshly deprotected *Compound 62*. The routine reagent for FMOC removal is 20% piperidine in DMF. *Compound 62* was treated in dry dichloromethane with an excess (20 eq.) of piperidine (99%) and monitored by T.L.C. Following complete deprotection the solution was basified and the acid chloride immediately introduced. Unfortunately, following a conventional work-up gave no material that could be identified as the required pentapeptide derivative, (*Scheme 3.34*).



Compound 62.

Scheme 3.34.

Chapter 4. Poly(ethylene) Glycol.

4.1. Strategy.

To provide the appropriately derivatised prepolymeric monomers various terminal groups were attached to PEG. The aim was to synthesise nucleophilic PEG endgroups which would be allowed to react with α,ω-glycidylether(peptide) functionalities in a polymerisation step. In order to encourage linear polymerisation a PEG terminal secondary amine was more preferable than a primary amine as the reaction of a glycidylether with a secondary amine is limited to only once. To accomplish this the PEG's were converted to α, ω-bis(chloroformate) with phosgene. Treatment with a suitable nucleophile [N-(BOC)sarcosine N-(2-aminoethyl)amide] gave the corresponding bis(carbamate) and deprotection of this compound with hydrogen chloride gave the required α,ω-bis(methylamino) derivatised PEG.

4.2. Introduction.

This chapter commences with a brief overview of the synthetic strategy that was adopted to activate the PEG in order to provide the appropriate functional groups for attachment to the peptide chain. The biological chemistry of a variety of PEG-enzyme and drug conjugates is discussed together with a review of the chemical literature concerning PEG. Finally there is a discussion on the work pertinent to the project.

4.3. Biological Chemistry of PEG.

To date, over 40 different proteins and other compounds have been modified with PEG (253) (*see Table 4.1*). Their uses vary considerable, although the majority of conjugates find their application within the biomedical and biotechnological spheres. In general, all the PEG-protein conjugates share the advantageous properties that are discussed in the introduction for macromolecular compounds. This section will focus on a number of examples which illustrate how the choice of coupling agent, molecular weight of the PEG, the percentage of groups modified and the specific properties of the protein being modified can alter the characteristics of the final PEG-adduct.

The effect of coupling PEG to enzymes has been well documented with extensive reviews being written (275). Studies on the modification of alkaline phosphatase with PEG (276) indicate that, if different coupling methods are compared, then it is apparent that some methods are more suitable at retaining enzyme activity than others. The monomethyl ether of PEG (M-PEG) with an average molecular weight of 5000 was activated using four electrophilic groups to couple covalently *via* reaction with protein nucleophilic centres. The derivatives were:- the cyanuric chloride [1]; the tresylate (2,2,2-trifluoroethanesulphonate) [2]; carbonyldiimidazole [3]; and the succinimidyloxycarbonyl [4]. Free and modified phosphatase activities were measured by following the enzyme catalysed hydrolysis of disodium *p*-nitrophenylphosphate to *p*-nitrophenol. With no modification the free phosphatase activity was assumed to be 100%. The percentage activity of the four types of activated M-PEGs, with high and low percentage modifications were compared to this control. The most active M-PEG protein was obtained by the use of the succinate conjugate [4] which gave a protein conjugate of comparable activity to the enzyme, even at a high modification level of 79%. The tresylate [2] and imidazole [3] gave a slightly less active

conjugate than the succinate whilst the cyanuric chloride method [1] gave extensive deactivation.

Table 4.1. PEG modified derivatives.

Protein To be modified.	Reference.	Protein To be modified.	Reference.
arginase	203	uricase	262, 254
L-asparaginase	202, 7, 254, 255	urokinase	263
batroxobin	204	adenosine deaminase	264, 254
blood coagulation factors IX	256	ovalbumin	201
elastase	257	ragweed pollen	265
α-galactosidase	211	mite	266
β-galactosidase	214	catalase	267
β-glucosidase	211	cholestrol oxidase	268
β-glucoronidase	206	chymotrypsin	269
haemoglobin	258	lipase	270, 210
immunoglobulin G	259	peroxidase	271
interleukin 2	191	papain	272
phenylalanine ammonia-lyase	260	trypsin	213
streptokinase	261	lipase	255
superoxide-dismutase	212, 254	urokinase	274
tryptophanase	273	plasminogen	274

Previous observations (205, 212) corroborate that the cyanuric chloride derivatives gives significantly more enzyme deactivation than do other activated PEGs. Boccu *et al* (277) mooted that this deactivation results from the close proximity of the two chlorines which can cause intramolecular cross-linking and appreciable deformation of the protein chain during the coupling process. It has long been established that a conformational change in the enzyme tertiary structure will adversely affect the affinity of the active site to the substrate resulting in a loss of turnover ability. It is interesting that difunctional PEG shows no increase in enzyme deactivation, suggesting that if the active sites of the difunctional PEG are separated by the long polymer chain, cross-linking is reduced, or, if it occurs, it introduces little deformation or rigidity into the protein chain.

The advantages of macromolecular conjugates has been discussed at length in the introduction and more pertinently in the preceding section. Listed below are just three, more detailed, examples from *Table 4.1* of how a drug carrier can be used to improve the clinical efficiency of drug delivery.

4.3.1. PEG Interleukin-2.

Human recombinant interleukin-2 (rIL-2) is currently being tested as an anti-cancer therapeutic agent. Unfortunately, the recombinant protein suffers from limited solubility at physiological pH and from immunogenic problems. The covalent attachment of PEG to rIL-2 produces a conjugate with bioactivity *in vitro* similar to that of unmodified rIL-2 (191) and gives a more hydrophilic molecule which is completely soluble at any pH. The enhanced solubility of PEG-rIL-2 eliminates the possibility of potential aggregates which may augment an immune response. This was corroborated when PEG-rIL-2 was tested in rabbits over an 8 month period and did not elicit an immune response (278). However, rabbits that had already developed neutralising anti-bodies after injection of only rIL-2 neutralised the

bioactivity of PEG-rIL-2 *in vivo*. This is suggestive that the conjugate does possess antigenicity but a reduced immunogenicity when compared to rIL-2. The conjugate can also display a marked increase in circulatory half life in mice when compared to free rIL-2. Two possible explanations for the decrease in plasma clearance could be due to the modification or shielding of the proteolytic sites of rIL-2 by PEG or the large increase in the size due to the hydration of the PEG within the conjugate, which reduces overall glomerular filtration.

Usually the covalent attachment of PEG to rIL-2 takes place by the random coupling of PEG to any or most of the lysines available, some of which may be in or near the active site. A recent report suggested that the covalent modification of four or more lysines resulted in a reduction in the bioactivity of the protein. This was probably caused by steric hindrance which prevented the binding of the conjugate to IL-2 receptors. Alternatively, there may have been some chemical modification at the active site. This prompted Goodson *et al* (276) to propose that PEGylation at a site distant from the active site would be preferable. Using mutagenesis techniques a 'handle' (*a cysteine thiol*) was engineered in a position distant from the receptor binding region of biological activity. Following reaction of a PEG-maleimide group with the thiol of cysteine, PEG-Cys3-rIL-2 was produced which had full biological activity.

The *in vivo* activity of a PEG-rIL-2 (Mw 95 000) species administered daily i.p has been investigated (191) in the murine tumour model, Meth A fibrosarcoma, and showed enhanced efficacy and reduced toxicity (8).

4.3.2. PEG-Superoxide Dismutase.

Superoxide dismutase (SOD) has been used as a novel anti-inflammatory metalloprotein drug in rheumatoid arthritis and has been modified with PEG (279). Huber

and Saifer (280) reported that unmodified superoxide dismutase was removed efficiently by the kidney from the serum of rats with a half life of 6 minutes, making effective treatment difficult in the clinic. In contrast, PEG-SOD remained in the circulation for 8 days and exhibited 51% of the native enzymatic activity when M-PEG 5000 was attached to 19 of the 20 lysine residues available on the enzyme (281). In addition, sensitisation work has demonstrated that the PEG-derivatised superoxide dismutase is a poor immunogen (282). This is in agreement with the previous studies whereby the extent of protein modification affects the enzyme activity but also confers protection on the conjugate. More recent research (283) on the toxicity of PEG-SOD evaluated in mice showed that the conjugates could be tolerated in large doses with no signs of toxicity. This is encouraging, particularly if repeated dosage regimes are to be employed for future therapy.

4.3.3 PEG-Granulocyte Colony Stimulating Factor.

The action of Granulocyte Colony Stimulating Factor (G-CSF) is exclusive to neutrophil generation. The availability of large quantities of biologically active recombinant material (rG-CSF) has made it possible to study it's use therapeutically. Recently, G-CSF has been administered to patients in order to accelerate their haematological recovery following high dose irradiation or drug induced neutropenia (low levels of neutrophils). Although therapy is successful, the factor is rapidly cleared from the circulation making daily injections necessary.

A comparative study of rG-CSF and PEG-rG-CSF on haematopoiesis (284) revealed that the response to PEG-rG-CSF was reduced when compared to that of the native protein. This paper suggested that the PEG molecules may either sterically inhibit the G-CSF receptor binding or that some of the lysine residues that were bound to the PEG were important for biological activity. Despite this, PEG modification of rG-CSF exhibited an

increased circulatory half life *in vivo* and consequently a sustained biological effect on the blood neutrophil level. This was beneficial as the effects of G-CSF are more positively influenced by the duration of G-CSF in the serum than by peak concentrations of G-CSF. Urinary excretion of rG-CSF is negligible and it was therefore assumed that the PEG was effectively shielding the potentially labile sites on the protein from circulatory proteases.

4.4. PEG Conjugates of Drugs.

The pharmacokinetic advantages displayed by PEG protein conjugates can be imparted to nonprotein moieties. The wide range of drugs which have been coupled to PEG is summarised in *Table 4.2.*

Table 4.2. A summary of PEG-Drug Conjugates.

Drug.	PEG. (Mw).	Ref.	Notes.
Penicillin V	PEG-OH 1900, 3000, 4000 polymeric	285 286	Penicillin remained active active.
Aspirin	PEG-OH PEG-OH	285 285	Same activity,↓ toxicity ↓ toxicity
Amphetamine	mPEG-SC	285	no reaction with DCC
Quinidine	PEG-COOH 1900, 3000	285	spacer BOC-Gly

Drug.	PEG. (Mw).	Ref.	Notes.
Atropine	PEG-OH	288	
	mPEG-NCO, 750, 1900, 2000, 5000.	285	hexamethylene spacer - Water soluble
	mPEG-COOH, 1900	285	no reaction with DMAP
Cephradine	polymeric	286	inactive
Procaine		289	
4-isobutyl phenyl - 2-propionic acid		290	anti-inflammatory

Drugs can be attached to PEG *via* several functional groups which are discussed in the next section. These include esters, amides, carbonates and urethanes. The polymer drug conjugates offers advantageous properties and a longer duration of activity due to their slow release.

4.5. Preparation of PEG Derivatives.

PEG has only two reactive groups (the hydroxyl groups at the ends of the chain) that are weak nucleophiles but they can be used directly, or after suitable functionalisation, for the covalent attachment of ligands. This process is often referred to as activation, and the product is sometimes called 'activated PEG'. With the development of new areas of application there is a growing demand for improved and versatile methods for the synthesis and characterisation of PEG derivatives. It is apparent that each of the activated forms of the polymer has features which can be considered an advantage or disadvantage, depending

on the goal sought. As a chemical short-hand the symbol PEG-R will be used to represent a difunctional PEG derivative R-$CH_2CH_2O(CH_2CH_2O)_nCH_2CH_2$-R.

There are three main approaches that can be used for the functionalisation of PEG:-

(1) activating the terminal hydroxyl group to a more active functional group, *e.g.* NH_2.

(2) reaction of the PEG with bi-functional components such that one of the functional groups reacts with PEG and the other remains active, *e.g.* N,N'-bis(phenylalanyl)-hexamethylene diamine.

(3) by preparing PEG ester derivatives through the generation of the fatty acid chlorides, followed by *in situ* condensation with PEG, *e.g.* RCOCl + PEG.

PEG is a hygroscopic material and the manufacturing process can introduce water into the product. Water can be a particular problem as this impurity and the PEG alcohols have similar nucleophilic reactivity. All commercial PEGs do contain some of this impurity with the lower molecular weight polymers have the highest amounts of water (approximately 1.0% for PEG 750). It is, therefore, prudent to dry PEG before laboratory use. This is often done by azeotropic distillation with benzene or, more commonly and safely, toluene in the Dean-Stark apparatus. Other methods include stirring the polymer under vacuum at 110 - 120°C. Any other purification is generally unnecessary as commercially obtained PEGs are often quite pure materials, having only trace (ppm) amounts of impurities such as dioxane, salts, and aldehydes. The concentrations of ethylene glycol and diethylene glycol, however, may vary considerably. Despite this, the molecular weight range is often reported as being narrow, for example PEG 8000 will vary by no more than 30 units in either direction (291). This is in contrast to other reports which suggest that the distribution of molecular weights of oligomers surrounding the mean value is often asymmetric. Approximately 95% of the mass of PEG 900 for instance is distributed between molecular weight 100 - 2000 with the distribution leaning towards the lower molecular weights (292). Experiments performed

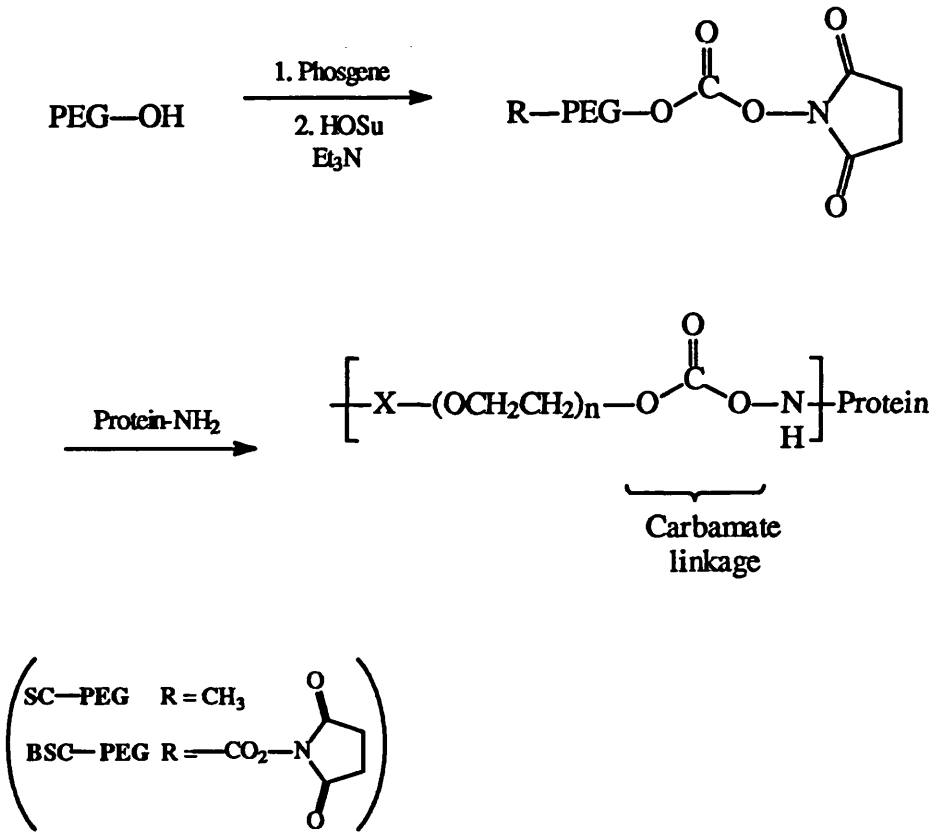
using these mixtures would make interpretation of the results unequivocal particularly if molecular weight and size were important factors, *e.g.* renal clearance studies, size standards for intestinal absorption. A prime practical consideration when using PEG - protein conjugates is to determine the optimum degree of substitution and PEG molecular weight needed to give the desired effect (on immunogenicity or partitioning, for example) without producing excessive protein deactivation. There appears to be no general rules in this regard, as effects vary greatly from system to system. Consequently, it is necessary to determine the effects of PEG molecular weight and degree of substitution in each case.

Proton NMR can be used for the qualitative identification of PEG functional groups although this technique has the disadvantage that the signals due to the repeating $O(CH_2CH_2O)$ units lie predominantly between δ 3.3 and δ 3.7 and obscure this area of the spectrum. Infrared spectroscopy (I.R.) is of some use particularly when searching for functional groups such as PEG acid chlorides. However, as with ^1H NMR, the problem is that the absorbing functional groups at the polymer terminals are present in low concentrations and thus give weak signals. Mass spectroscopy techniques are generally of little value as the parent ion is not detected and small fragment ions of low mass are often produced. Classical qualitative analysis can be useful in establishing end group functionality.

4.5.1. Electrophilic Activation of PEG.

There has been a great deal of interest in electrophilic PEGs for coupling to free amino groups of proteins and these same derivatives are potentially useful for reaction with other nucleophiles. The literature suggested that one route that had been successfully used to derivatise PEG was by nucleophilic substitution on electrophilic PEG derivatives. In this section these electrophilic derivatives are considered.

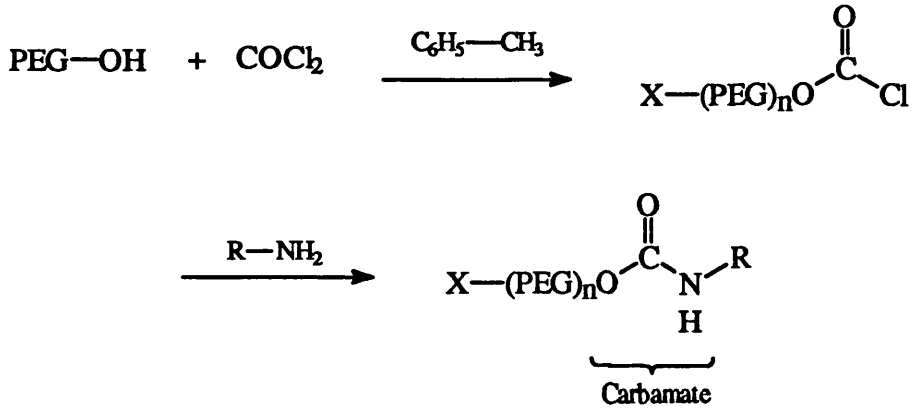
(A) In 1991 Zalipsky (293) described the preparation and use of a new protein modifying reagent methoxypoly(ethylene glycol)-N-succinimidyl carbonate (SC-PEG) and the bifunctional analogue poly(ethylene glycol)-bis-(N-succinimidyl carbonate) (BSC-PEG), (*Scheme 4.1*).



The protocol Zalipsky employed was an overall improvement on the existing methods of protein conjugation as carbamate linkages between amino groups of a protein and PEG provide a stable attachment, more resistant to hydrolytic cleavage (294). N-Hydroxysuccinamide (HOSu) released during polypeptide modification using BSC-PEG does not show affinity towards proteins and can be readily removed from the reaction solutions. An additional advantage of this activated PEG is that those reactive functional groups that do not react with amino groups of a protein or reagent undergo hydrolysis producing HOSu,

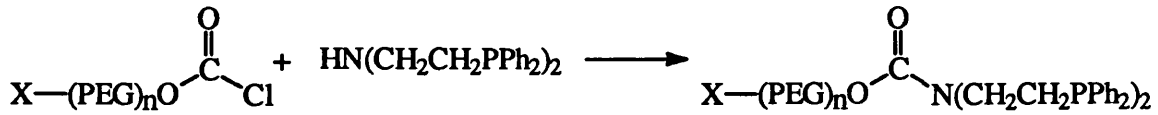
CO_2 and hydroxy terminated polymer. This feature is of particular importance in the case of the bifunctional BSC-PEG.

Similarly, PEG chloroformate has been prepared and treated with an amine to make a carbamate by Takerkart (333), (*Scheme 4.2*). The use of PEG-phenyl carbonate derivatives for the preparation of carbamate linked PEG-proteins was reported by Veronese (294). The main drawback of this approach lies in the toxicity of hydrophobic phenol residues (*p*-nitrophenol or 2,4,5-trichlorophenol) and their affinity for proteins.



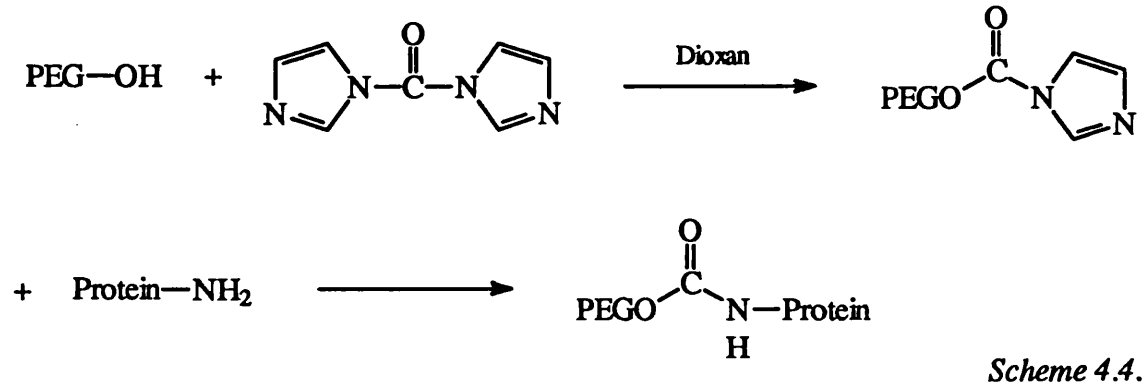
Scheme 4.2.

Takerkart, by attaching *p*-aminobenzaldehyde (which is a trypsin inhibitor) used the chloroformate in the affinity phase partitioning of trypsin. It is also reactive toward other nucleophiles, *e.g.* reaction with alcohols gives carbonates (289). Nuzzo used this active derivative for preparing phosphine derivatives for binding transition metal catalysts (295), (*Scheme 4.3*).



Scheme 4.3.

In one alternative route, Beauchamp treated PEG with carbonyldiimidazole to produce an activated electrophilic derivative, which is subject to nucleophilic attack by lysine amino groups (212), (*Scheme 4.4*). The PEG is attached to a protein *via* a carbamate linkage and this intermediate is said to give little deactivation upon reaction with proteins. However, the polymer activated in this manner was not very reactive and therefore very long reaction times (48 - 72h at pH 8.5) were required to achieve sufficient modifications.



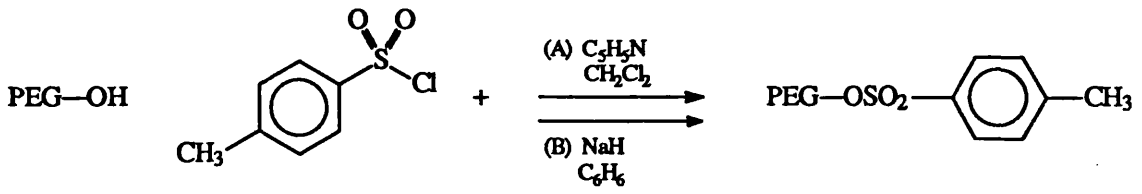
Scheme 4.4.

(B) PEG Chloride and PEG bromide (334) have been prepared by reaction with thionyl chloride or bromide in toluene, (*Scheme 4.5*). The bromide derivative appears more stable than the chloride, with samples remaining unchanged for at least a year.

Scheme 4.5

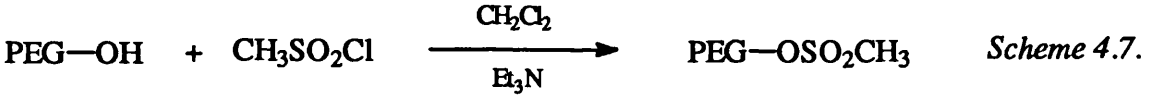
Bayer (296) noted that the preparation of the chloro derivative can be simplified by omitting solvent and simply running the reaction in an excess of thionyl chloride, which is subsequently removed by distillation.

Pillai *et al* (297) and Harris (298) have prepared the tosylate by a common route involving pyridine as an acid scavenger, (*Scheme 4.6(A)*).



Scheme 4.6.

These authors have reported (298) that this procedure produces a reduction in PEG molecular weight of approximately 30% and that this compound decomposes slowly on storage. A preparation without chain cleavage is provided by eliminating the pyridine and preparing the PEG alkoxide by reaction with sodium hydride (*Scheme 4.6(B)*). This result indicates that pyridine hydrochloride may be the agent causing chain cleavage in the first tosylate preparation. Further evidence that this may be the case is indicated by preparing the tosylate (299) and mesylate (300) without cleavage by use of triethylamine as acid scavenger, (*Scheme 4.7*).



Scheme 4.7.

A further review or the literature reveals other candidate electrophiles which may also be suitable, namely the nosylate and brosylate, (*see figures 4.1 and 4.2*).

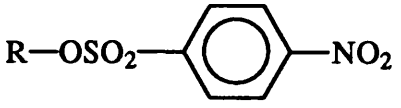


Fig. 4.1. Nosylate

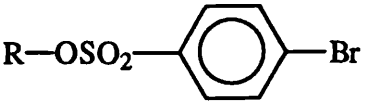
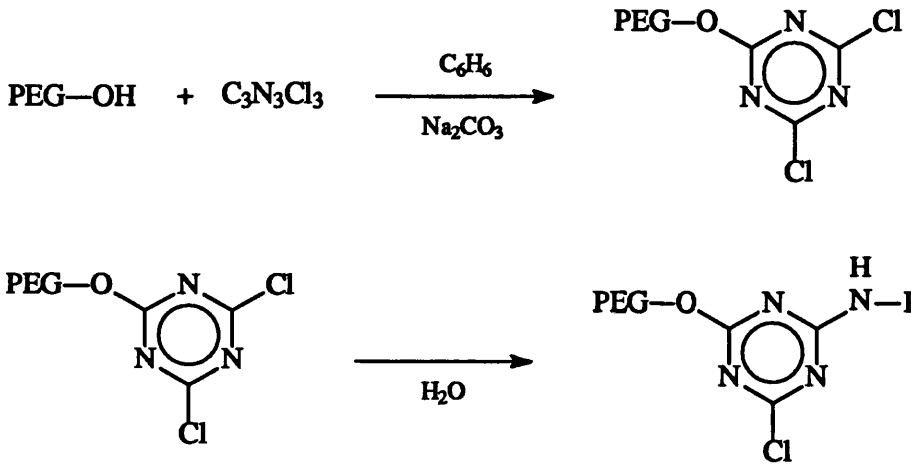


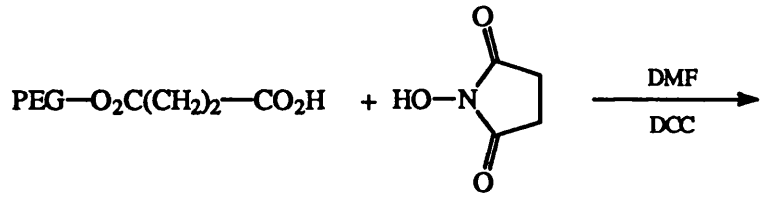
Fig. 4.2. Brosylate

(C) The most frequently applied route to cyanuric chloride activation of PEG is that of Abuchowski and Davis in which the PEG-cyanuric chloride derivative is allowed to react with the protein (194, 301, 259), (*Scheme 4.8*). This approach suffers from disadvantages such as the toxicity of cyanuric chloride and limited applicability for modification of proteins having essential cysteine or tyrosine residues as manifested by their loss of activity (302, 199, 212). Despite this there has been some success (197) for the covalent attachment of bovine serum albumin to PEG. The first of the two chlorines on this derivative is substituted by protein amino groups in about one day. The second site is much less reactive, although there does appear to be some reaction (277).

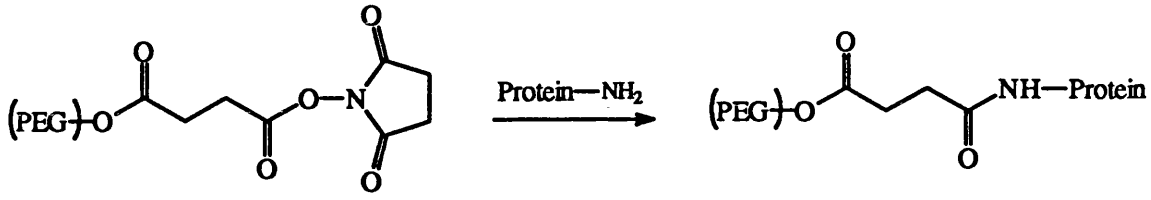


Scheme 4.8.

(D) Currently a common form of activated PEG is the succinate-N-hydroxysuccinimide ester, (SS-PEG) (199). This "active ester" has been examined by two groups (303, 304), (*Scheme 4.9*).

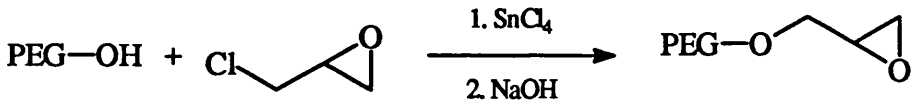


Scheme 4.9.



It reacts with proteins in short periods of time under mild conditions producing extensively modified conjugates with well preserved biological activity. However, the ester linkage between the polymer and the succinic acid residue has limited stability in aqueous media. An early version of this approach is the synthesis by Royer (305) of the 1,2-bis(succinimidyl succinate) of ethylene glycol.

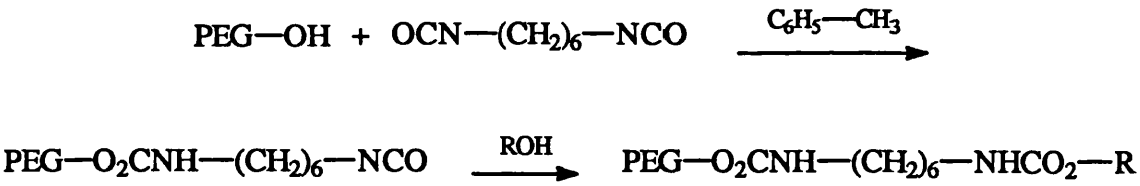
(E) Pitha has prepared PEG epoxides by reaction of PEG with epichlorohydrin in the prescence of the Lewis acid tin (IV) chloride followed by ring closure with sodium hydroxide (306) (*Scheme 4.10*).



Scheme 4.10.

This derivative is sufficiently electrophilic to react with both primary and secondary hydroxyl groups of polysaccharides.

(F) The isocyanate is a reactive electrophilic derivative which can be reacted with amines or alcohols to yield ureas or carbamates (307), (*Scheme 4.11*).



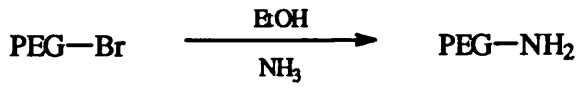
Scheme 4.11.

Zalipsky (285) found that dibutyltin dilaurate was an effective catalyst for the coupling reaction although it was noted that the PEG isocyanate decomposed upon standing and should therefore be freshly prepared before use. Interestingly, the carbamate linkage appears to be useful as a labile linkage for the controlled release of drugs (285).

4.5.2 Nucleophilic Activation of PEG.

PEG amines are important as intermediates in the synthesis of other derivatives, and several PEG amines are themselves useful in direct application.

(A) Buckmann and Johansson have described two direct syntheses for primary PEG amines, the most useful form for subsequent derivatisation (303, 308), (*Scheme 4.12*).



Scheme 4.12.

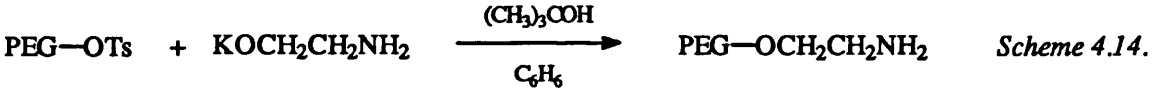
The first method is direct and produces 100% substitution by the use of gaseous ammonia. The second method is easily applied and although a primary and a secondary amine are produced, the primary is much more reactive. Since the hexane 1,6-diamine is difunctional,

there is the possibility in this reaction of interconnecting PEG chains. Johansson has found this to occur if the molar ratio of bromide to amine is 0.19 but not if it is 0.05.

Zalipsky has described an effective three step synthesis of PEG amine, *via* the azide which gives an 80% yield of completely aminated product (285), (*Scheme 4.13*).

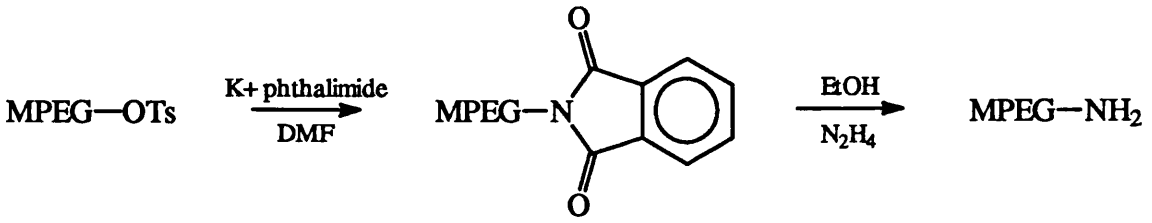
Scheme 4.13.

Another route is provided by the method of Kern (309), (*Scheme 4.14*), using the activated PEG-OTs derivative.



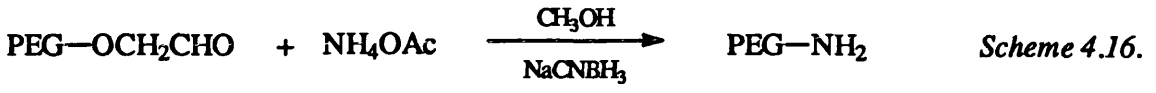
Scheme 4.14.

Mutter (310), Geckeler (311) and Ciuffarin (312) have applied the Gabriel phthalimide-hydrazine sequence to the preparation of PEG amine, (*Scheme 4.15*). This has two steps and leads only to a primary amine, but it has been used with success in the many examples of peptide synthesis by Mutter and coworkers.



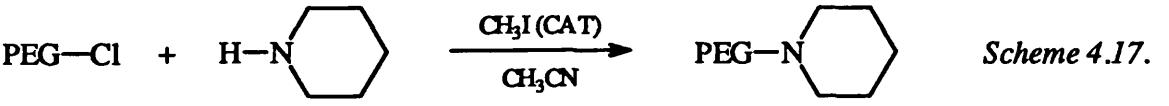
Scheme 4.15.

PEG amine can also be prepared by reductive amination of PEG aldehyde with ammonium acetate (313), (*Scheme 4.16*), this method gives essentially complete substitution with no chain cleavage.



Scheme 4.16.

A preparation of PEG secondary amine has been published by Suzaki *et al* (314) who have carried out displacements on PEG tosylate and chloride with amines (*Scheme 4.17*).



Scheme 4.17.

4.6. Investigations of Routes to α,ω-Bis (methylamino)PEG Derivatives.

Although the literature search reveals that a considerable amount of work has been done on PEG, the availability of a range of commercial products is still awaited. The reaction of α,ω bis(amino) PEG with α,ω-bis(glycidyl)peptides was chosen as the polymerisation strategy towards the alternating PEG-peptide linear polymer. To provide the appropriately derivatised monomer, a number of experimental strategies were investigated.

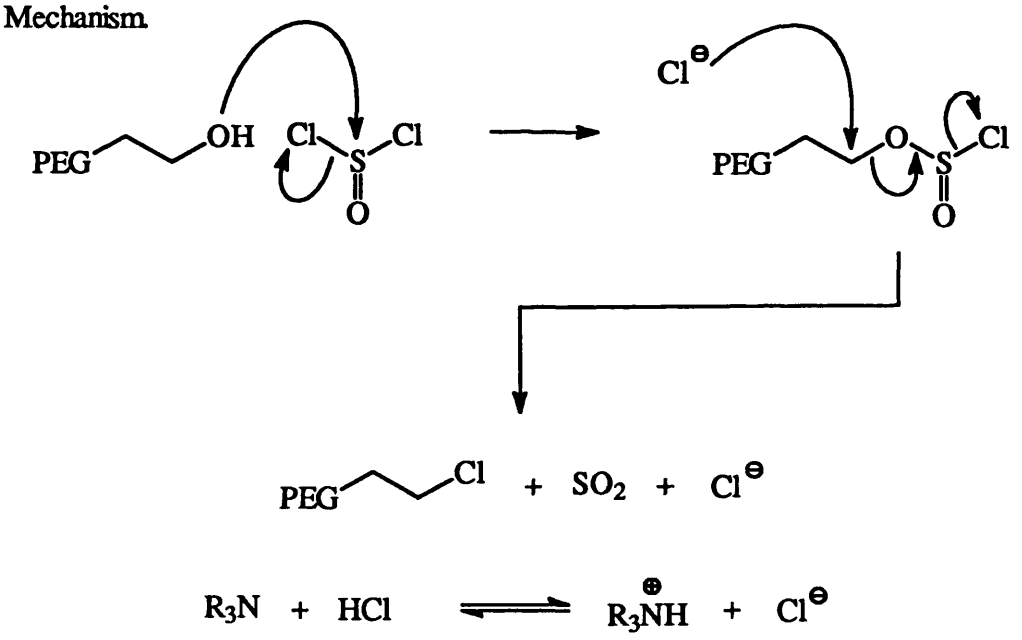
4.6.1. The alkyl halides. Tosylate esters.

Many α,ω-bis(amino)PEGs have been prepared by substitution of α,ω-dichloro PEGs with ammonia at elevated temperatures and pressures. However, a more straightforward attempt

was made to adapt Bayer's synthesis (296) of α,ω-dichloro PEGs to hexa(ethylene glycol), a model oligomer of defined chain length. However, treatment of hexa(ethylene glycol) with refluxing thionyl chloride gave no material which could be identified as the required dichloro derivative, (*Scheme 4.18*).

Scheme 4.18.

A mechanistic scheme for this proposed transformation is shown in *Scheme 4.19* whereby a tertiary amine R_3N, *e.g.* triethylamine or pyridine is used to catalyse the reaction by forming a chloride ion from the HCl produced.

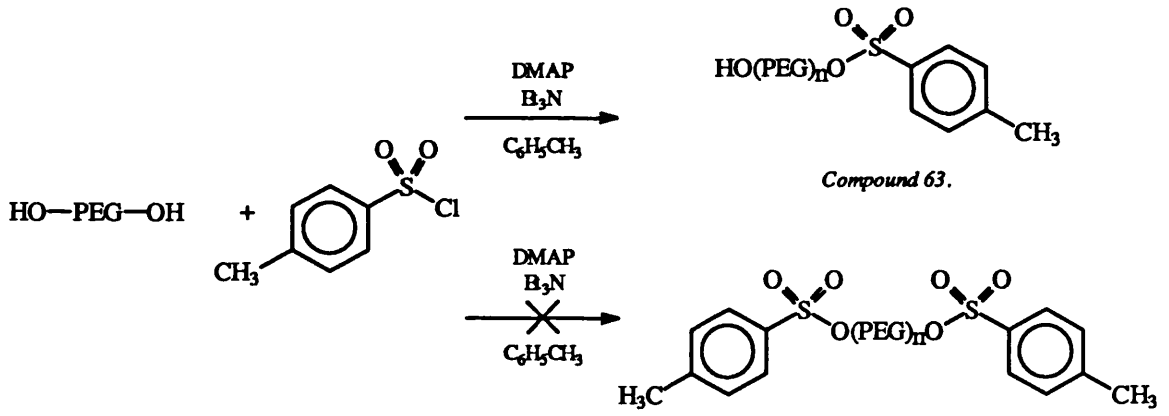


Scheme 4.19.

Since tosylate is a much better leaving group than chloride, synthesis of the α,ω-bis(tosylate) of the model hexa(ethylene glycol) was investigated. These compounds react with primary and secondary alcohols, generally in the prescence of a tertiary amine, *e.g.* pyridine to

produce sulphonate esters. The hexa(ethylene glycol) (Mw 282) was dried by azeotropic distillation of the water with toluene using a Dean and Stark trap. The diol was treated with tosyl chloride in the prescence of triethylamine (a tertiary amine base) and a catalytic amount of 4-(dimethylamino)pyridine (DMAP, a nucleophilic catalyst). Conventional work up gave only a multi-component intractable gum but direct application of the crude reaction mixture to a chromatography column and subsequent elution permitted the isolation of a single product in moderate yield.

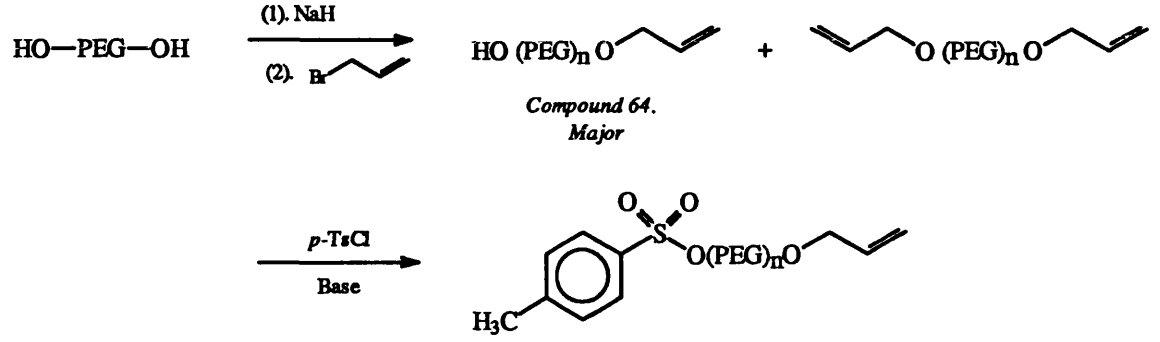
This material was identified as the mono-tosylate by proton NMR spectroscopy, (*Compound 63*) (*Scheme 4.20*). Comparison of the integrals of the aromatic methyl singlet at δ 2.45; the aromatic signals at δ 7 - δ 8 and of the PEG CH_2 cluster at δ 3.67 - δ 4.15 showed a 1 : 1 ratio of PEG to tosylate.



Scheme 4.20.

An alternative tosylation was attempted according to the method of Dellaria (321). This method was adapted to investigate the feasibility of activating penta (ethylene glycol) with a mono tosylate whilst protecting the alcohol with an olefin. The synthesis began with the mono alkylation of the sodium anion of penta (ethylene glycol) with allyl bromide. After one hour the reaction was acidified and the crude product flash filtered through a silica plug

to give a product in good yield, (*Scheme 4.21*). The identity of the product was confirmed by ^1H NMR which revealed characteristic allylic peaks at 4.02 - 5.90 which integrated correctly for *Compound 64*.

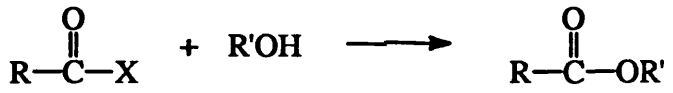


Scheme 4.21.

Tosylation of *Compound 64* was achieved by combining an excess of *p*-tosyl chloride with triethylamine and a catalytic amount of DMAP. A conventional work-up followed by column chromatography provided a product (*Compound 65*) in moderate yield which gave a parent ion at *m/z* 433 in the mass spectrum. Confirmation of the required product was corroborated by proton NMR which showed the methyl (tosyl) group at δ 2.45 and the aromatic signals at δ 7.34 - δ 7.79.

4.6.2. The Alcoholysis of Acyl Halides.

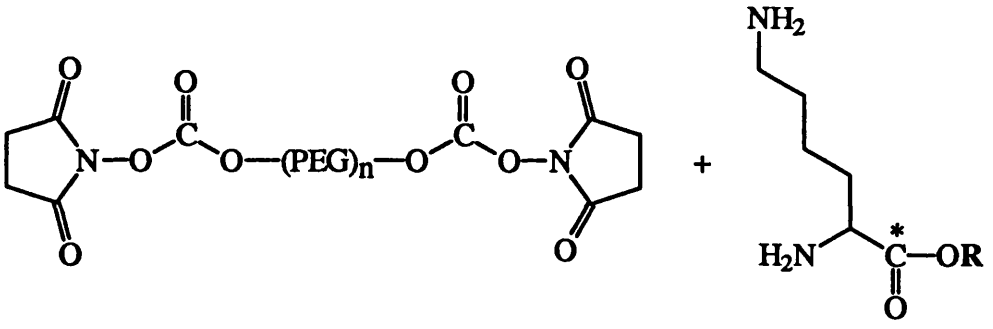
The reaction between the acyl halides and alcohols is the best general method for the preparation of esters (315), and it has been used successfully for the esterification of PEG. A general formula is given, (*Scheme 4.22*).



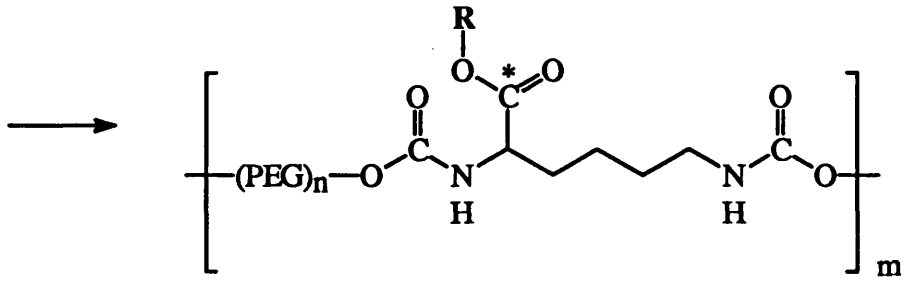
Scheme 4.22.

The reaction is of wide scope as both R and R' may be primary, secondary or tertiary, alkyl or aryl. An important example is the preparation of carbobenzyloxy chloride ($PhCH_2OCOCl$) from phosgene and benzyl alcohol. This compound is widely used for the protection of amino groups during peptide synthesis.

Zalipsky (293) and Nathan *et al* (316) reported an efficient one-pot procedure for the preparation of succinimidyl carbonate (SC) activated PEG which was repeated successfully in our laboratory using hexa(ethylene glycol). Previously Zalipsky and Nathan had used this procedure to prepare an activated 'pre-polymer' in essentially quantitative yields. They were able to treat bis succinimidyl carbonate (BSC)-PEG with L-lysine ethyl ester a poly (ether carbamate) consisting of strictly alternating units of PEG and L-lysine ethyl ester, was obtained, (*Scheme 4.23*).

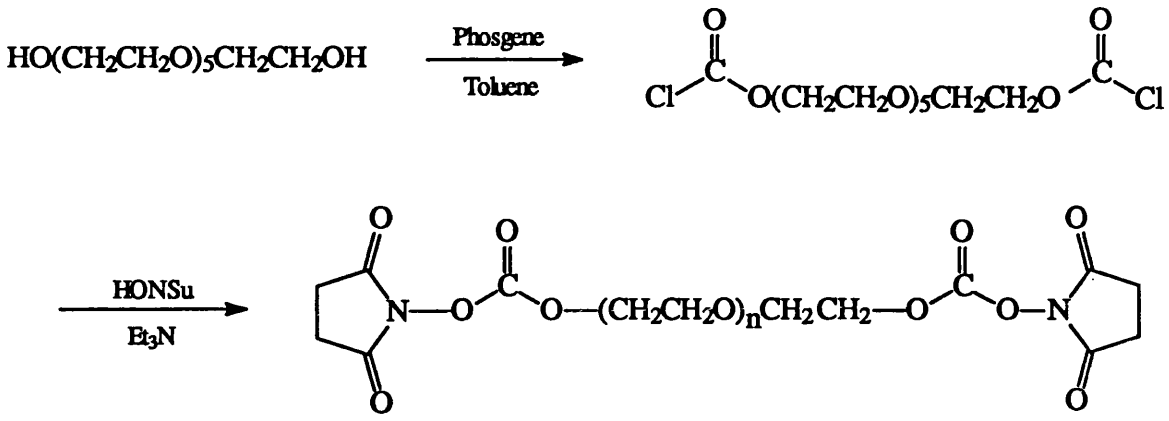


Scheme 4.23.



It was therefore hypothesised that these same activated groups should be capable of reaction with a secondary nucleophile.

PEG α,ω-di(chloroformate) was generated from the diol by the treatment of hexa-(ethylene glycol) with excess phosgene (10 eq.) in a mixture of dry toluene and dichloromethane (*Scheme 4.24*). Due to the hazardous nature of the reagents involved and because the intermediate was known the bis(chloroformate) was not characterised but instead preliminary experiments were initiated on the derivatised hexa(ethylene glycol).



Compound 66.

Scheme 4.24.

The polymeric bis(chloroformate) was allowed to react with N-hydroxysuccinimide *in situ* to give a crude product which after column chromatography, afforded the corresponding bis (succinimidyl)carbonate (BSC-PEG) derivative (*Compound 66*), in moderate yield.

Following the successful synthesis of BSC-PEG the problem of treating the functionalised PEG to form a nucleophilic centre became more obvious. Further work (discussed in Chapter 5) had been completed on an analogue which was able to react with the bis(epoxide) in a similar fashion to PEG. In order to investigate the feasibility of a reaction between an amine on PEG and the oxiranyl group we considered 2-methoxyethyl-amine (*Figure 4.3(A)*) as a model. This compound behaved in a similar fashion to PEG but no polymerisation was possible because it was mono-functional. Further consideration of this reaction suggested that the amine group may, after the initial reaction be capable of a further reaction as a nucleophilic amine would be formed giving rise to cross-linking. It was therefore considered better to use 2-methoxy-N-methylethylamine (*Figure 4.3(B)*) which, as a secondary amine would be capable of a single reaction and therefore be a model for a linear polymer. Of course using 2-methoxy-N-methylethylamine further reaction remains

impossible due to the methoxy group and to initiate a successful polymerisation, a polyoxyethylene (bis) amine was required.

(*A*). (*B*).

Fig. 4.3.

4.6.3. The N-(BOC)sarcosine N-(ethane-1,2-diamine) formation.

It was anticipated that the electrophilic glycidyl ether (peptides) would be able to react with the PEG α,ω-bis(amine) during the final polymerisation step. A strategy was devised using sarcosine which provided a preformed secondary amine on an amino acid, (*Figure 4.4*).

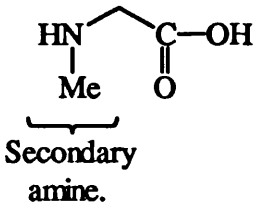
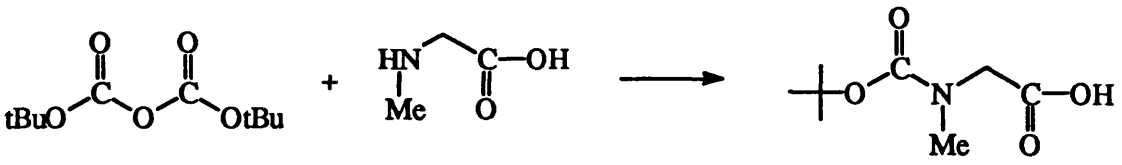


Fig. 4.4. Sarcosine

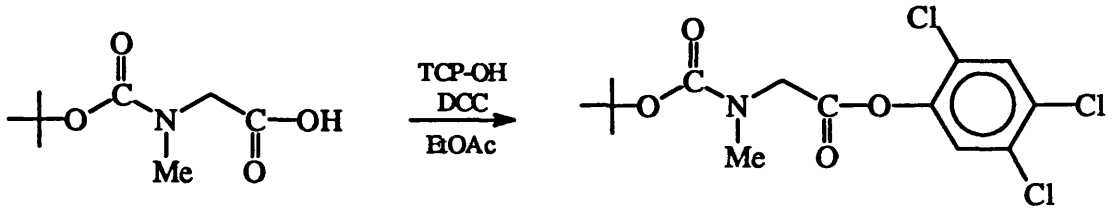
A conventional *t*-butyl (BOC) reaction provided the protected amine (*Compound 67*) in good yield which melted at the stated literature value, (317) (*Scheme 4.25*). Characterisation with ^{1}H NMR showed that following protection the methyl peak is transformed from a doublet to a singlet with the removal of the proton on the amine. An extra singlet was frequently seen with ^{1}H NMR in the spectrum of the product at δ 1.48 and δ 2.95 corresponding to the BOC peak and methyl peak, respectively.



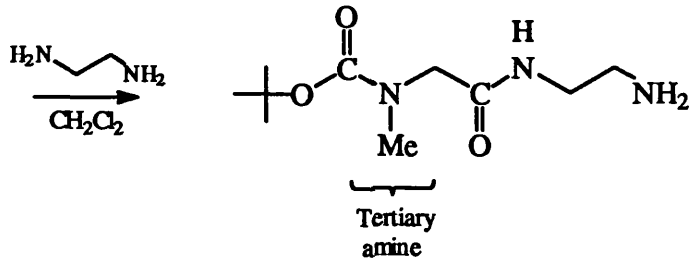
Compound 67.

Scheme 4.25.

Conversion of the protected amino acid to an active ester was achieved with 2,4,5-trichlorophenol *via* dicyclohexylcarbodiimide (DCC) coupling. The two protons carried on the trichlorophenyl ester allow the progress of the coupling to be monitored by ^{1}H NMR and the product was afforded in good yield, (*Scheme 4.26*), (*Compound 68*).



Compound 68.



Compound 69.

Scheme 4.26.

The bifunctional, ethan-1,2-diamine was prevented from reacting with multiple active ester units by adding the activated ester dropwise into a large excess of the nucleophile. Statistically this reduces the chance of a nucleophile reacting with two electrophiles and the formation of the desired product is favoured. The excess amine was removed during the

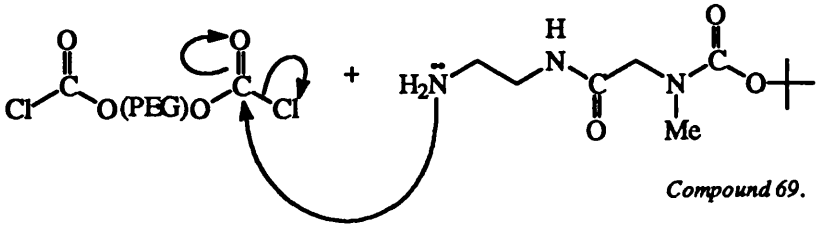
work up by washing the organic phase with a small amount of water in which the ethan-1,2-diamine was readily soluble. Evaporation of the organic phase gave a product (*Compound 69*) in good yield which was analysed with proton NMR and mass spectroscopy. Comparison of the BOC singlet at δ 1.40, of the methyl singlet at δ 2.80, of the amide singlet at δ 7.90 and the broad peak of the amine at δ 4.00 suggested the product was BOCsarcosine N-(2-aminoethyl)amide. This was corroborated with the observation of the M + H ion peak at *m/z* 232 in the mass spectrum.

In order to attach *Compound 69* to PEG and provide the α,ω-bis(methylamino)PEG the weakly nucleophilic hydroxy groups were converted to the chloroformate. The PEG bis(chloroformate) was prepared using a method adapted from Zalipsky (213) and isolated free from any contamination with phosgene. A molar excess of triethylamine, a catalytic amount of DMAP and an excess of N-(BOC)sarcosine N-(2-aminoethyl)amide (*Compound 69*) ensured that as the bis(chloroformate) was added in a dropwise fashion it would be allowed to react immediately, (*Scheme 4.27*). The acid labile BOC protecting groups were not compromised as the presence of triethylamine prevented the pH from becoming too low. The crude product was washed with water and weak acid before a moderate yield of product (*Compound 70*) was assigned with ^{1}H NMR. A large broad singlet which integrated to 18 reflected the prescence of the two *t*-butyl protecting groups at δ 1.38 and five sets of signals were assigned to the amides with the methyl protected amides positioned at the same shift value.

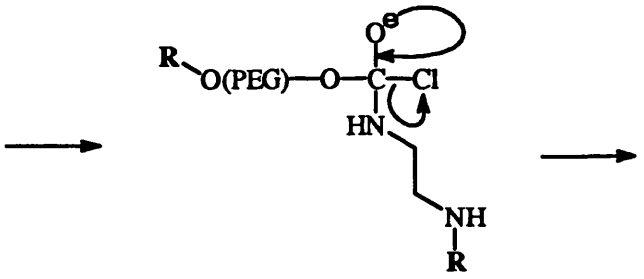
Deprotection of the sarcosyl protecting groups was effected with an excess of hydrogen chloride and the reaction was followed by thin layer chromatography (T.L.C.) using ninhydrin to visualise the newly exposed primary amines. Characterisation studies by ^{1}H NMR on the hydrochloride salt (*Compound 71*) of the product showed the characteristic broad singlet at 1.38 ppm for *t*-butyl protons was absent, but a multiplet had been formed

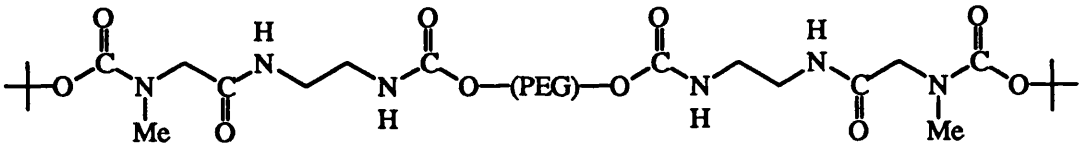
down field at δ 9.00 which was assigned to the α,ω-bis(amines). Mass spectroscopy revealed a mother ion peak at *m/z* 553 which corroborated that the product had been formed.

Mechanism of the acyl halide and N-(BOC)sarcosine N-(ethane-1,2-diamine) reaction.

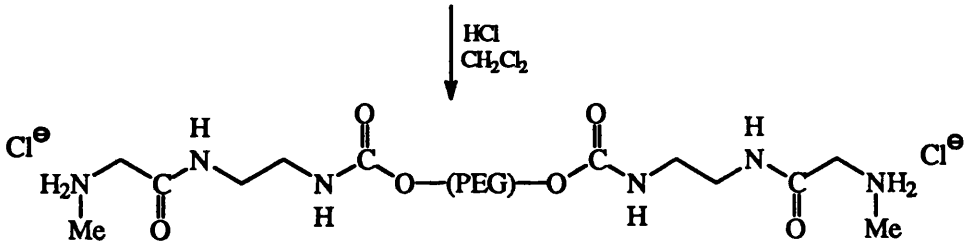


Compound 69.





Compound 70.



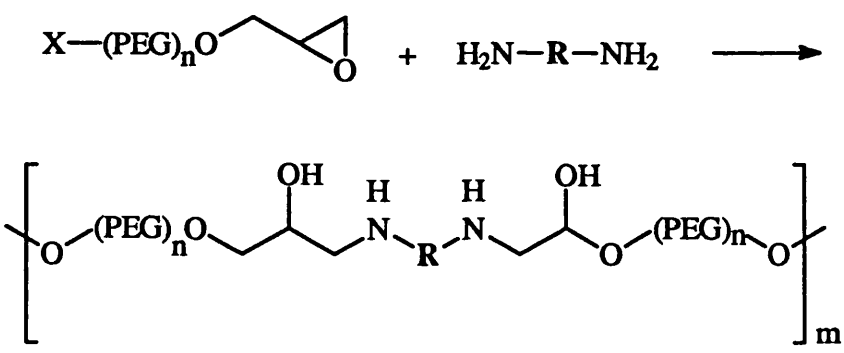
Compound 71.

Chapter 5. The Polymerisation Process.

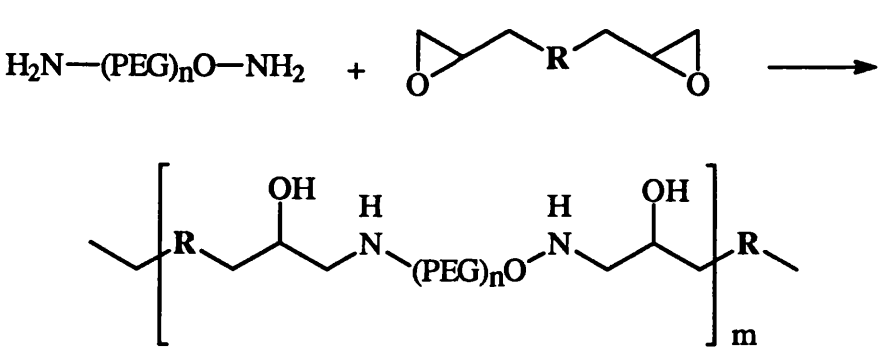
5.1. Strategy

The appropriately derivatised PEG monomers and peptide chains for copolymerisation could be generated in a number of ways. One suitable strategy was to synthesise various di, tri, tetra and penta peptides with orthogonal side chain protecting groups bearing either a nucleophilic amine at each end or an electrophilic glycidyl ether at each end. These functional groups could be treated with PEG monomers bearing α,ω-bisoxiranes (*Scheme 5.1(A)*) or α,ω-bisamines (*Scheme 5.1(B)*), respectively, to form the final polymers.

(A).



(B).



R = peptide. *Scheme 5.1.*

A series of peptides were prepared bearing α,ω-bis glycidyl ethers. Various PEG monomers were derivatised with a secondary amine and allowed to react with the glycidyl ethers in a polymerisation process.

Through the careful selection of the length of PEG monomers, the size and molecular weight of the copolymers could be adjusted to enhance the biodistribution of the conjugate through passive targeting. The polymerisation process should succeed in producing strictly alternating copolymers of PEG and an oligopeptide chain. The peptide sequences are labile to intracellular lysosomal enzymes, and provide an opportunity to make the polymer biodegradable. In addition, the copolymers should retain the desirable properties of PEG while providing an opportunity to attach reactive pendant groups (the carboxylic acid groups of glutamate) at predetermined intervals along the polymer backbone.

5.2. Introduction

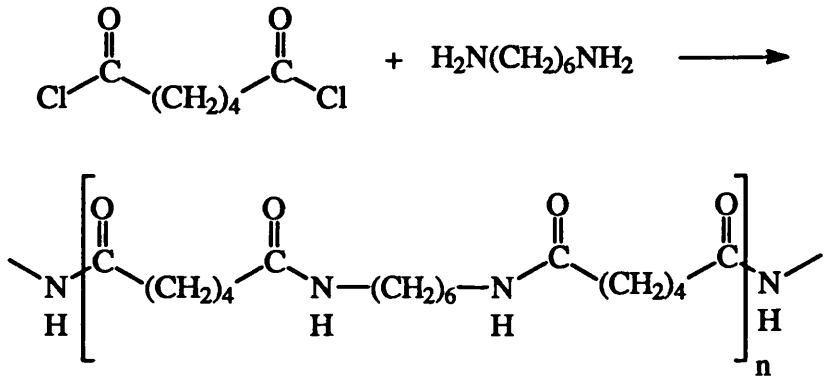
The definition of a polymer (from the greek "polys", many, and "meros", parts) is a macromolecule made up of repeating structural units. The reaction that unites the small molecules (monomers) into a polymer is called polymerisation. Examples of naturally-occurring polymers include starch, cellulose, proteins and nucleic acids.

Synthetic polymers can be divided into two main classes, depending on their method of synthesis. Addition (chain growth) polymers are formed by a reaction in which the monomer units add to one another. The most common types are vinyl polymers, and examples include polyethylene, polypropylene and polystyrene.

Copolymers are prepared from the copolymerisation of two or more monomers. Since the ratio of the monomers can vary widely, copolymers for specific uses can be tailor

made. Condensation (step growth) polymers are formed by reactions of difunctional or polyfunctional molecules, such as glycols, diamines and dicarboxylic acids, to form polyesters and polyamides, through the elimination of small molecules such as water, alcohol or ammonia. Nylon (polyamide), Dacron (a polyester) and polyurethanes are common examples of polymers formed by this type of reaction.

The polymerisation strategy used in this project is based upon similar reactions. For example reaction of a dicarboxylic acid or diacyl chloride (adipoyl chloride) with a diamine (hexamethylenediamine) leads to the formation of a linear polymer, namely nylon (*Scheme 5.2*).

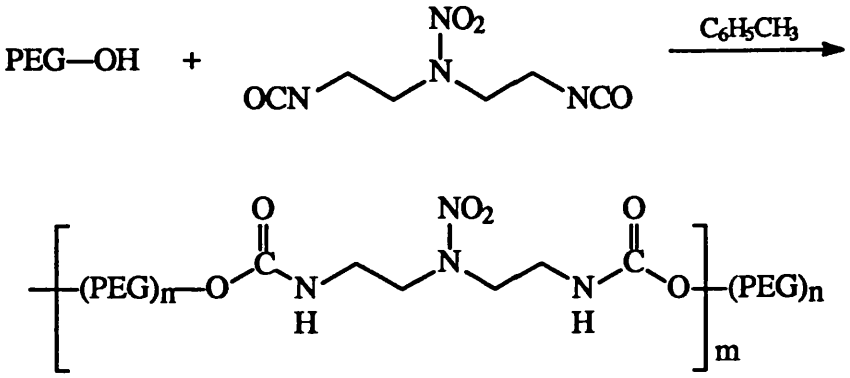


Nylon 6,6 *Scheme 5.2.*

It should be noted that this type of polymer is not the product of a free radical chain reaction, but results from a simple repetition of a normal reaction of the polar type. The only requirement is polyfunctional compounds.

5.3. PEG as a Monomer for Polymerisation:- A Review.

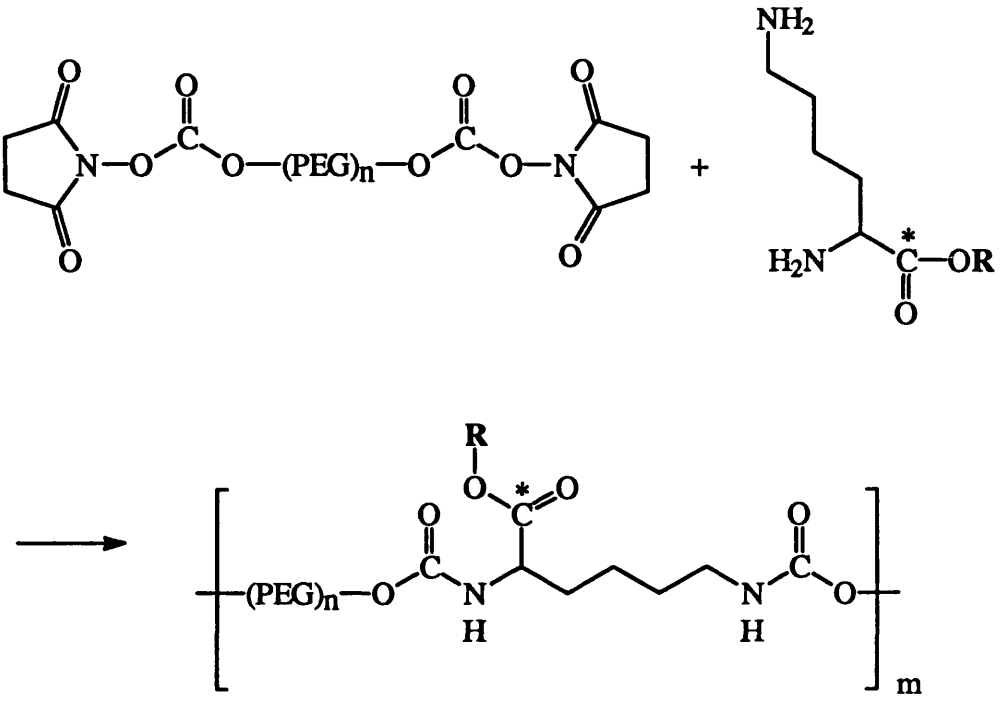
In the past, PEG chains have been copolymerised with a variety of difunctional comonomers. Block copolymers of PEG and two isocyanates, 3, 5-diisocyanato-benzyl chloride or 3-nitro-3-azapentan-1,5-diisocyanate have been prepared by Bayer for use as soluble protecting groups in peptide synthesis (318), (*Scheme 5.3*).



Scheme 5.3.

These materials carry functional groups at defined distances all along the chain and thus a high degree of substitution is possible along the chain.

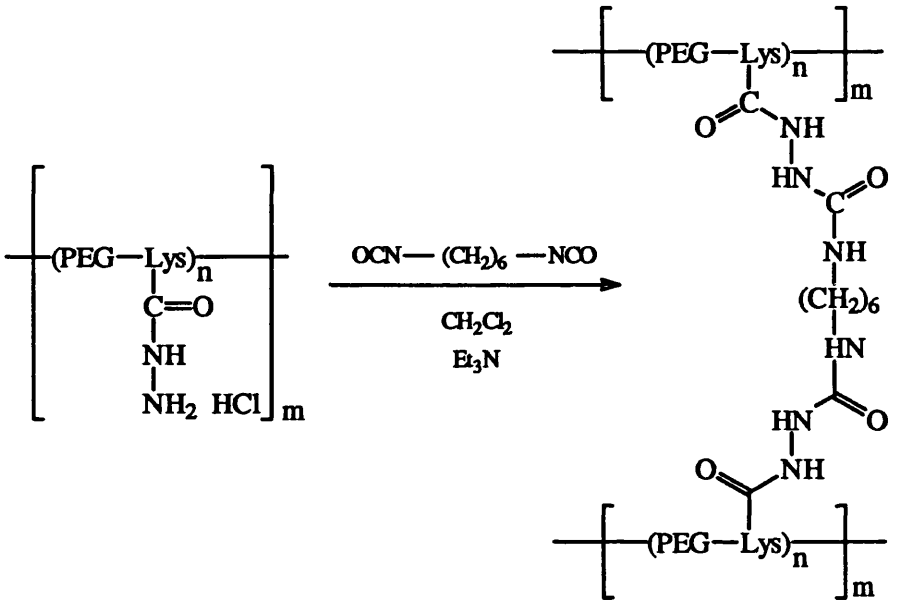
Water soluble poly(ether urethanes) have been prepared by Nathan *et al* (316) through the copolymerisation of bis(succinimidyl)carbonate derivatives of PEG with lysine (L-Lys) in a strictly alternating fashion. The resulting copolymers have physical properties similar to those of PEG while the carboxylic acid groups of L-lysine provide multiple pendant groups along the polymer backbone for further functionalisation.(see *Scheme 5.4*)



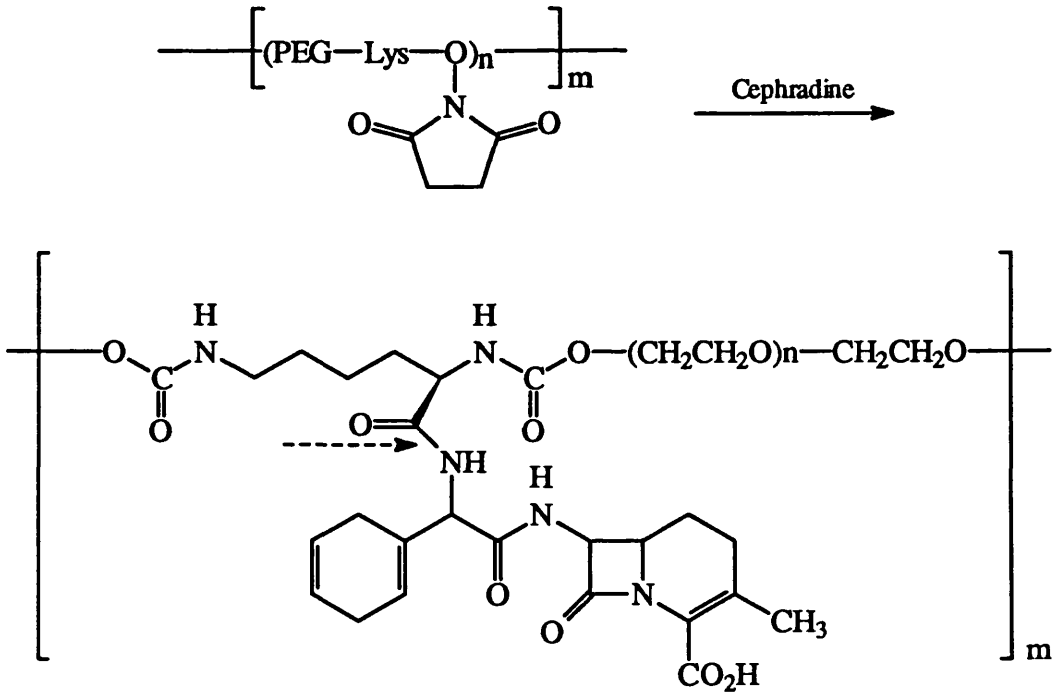
Scheme 5.4.

Nathan *et al* (316) also utilised the pendant lysyl carboxylic groups in cross linking reactions leading to amide cross links, new acyl semicarbazide cross links (*Scheme 5.5*) and hydroxyethyl acrylate and hydroxyethyl methacrylate-derived cross links. The resulting hydrogels had favourable physicomechanical properties and were non toxic, which may encourage their application as biomaterials.

A further pioneering paper by Nathan (286) in 1993 reported that activated pendant carbonyl groups of poly(PEG-Lys) had been used as anchors for the covalent attachment of penicillin V and cephradine (see *Scheme 5.6*), two clinically used antimicrobial agents. The versatility of this system was demonstrated by the preparation of conjugates having antibiotic ligands linked *via* a biostable or biodegradable linkages to the carrier. The dotted arrow indicates the most probable site of hydrolytic cleavage.



Scheme 5.5.

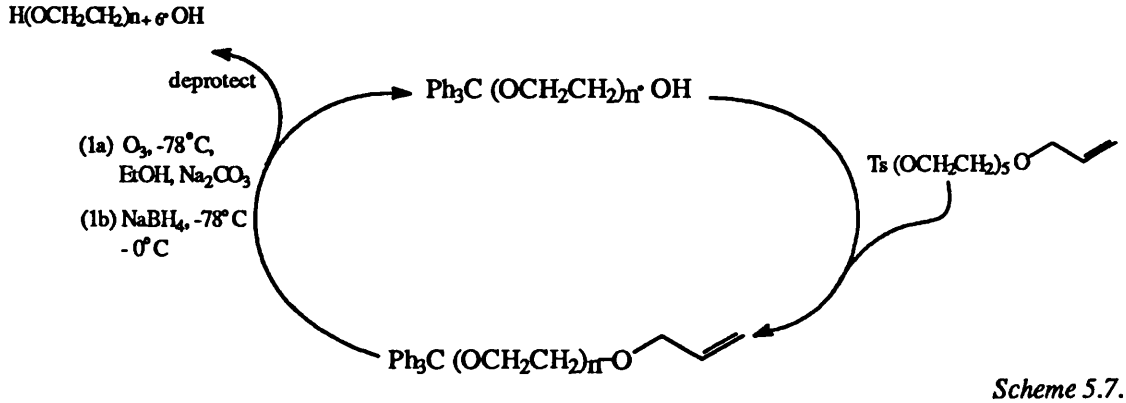


Scheme 5.6.

The chemical versatility of this new class of polymers for the preparation of various drug conjugates represents a significant advantage over previously reported drug-carrier systems.

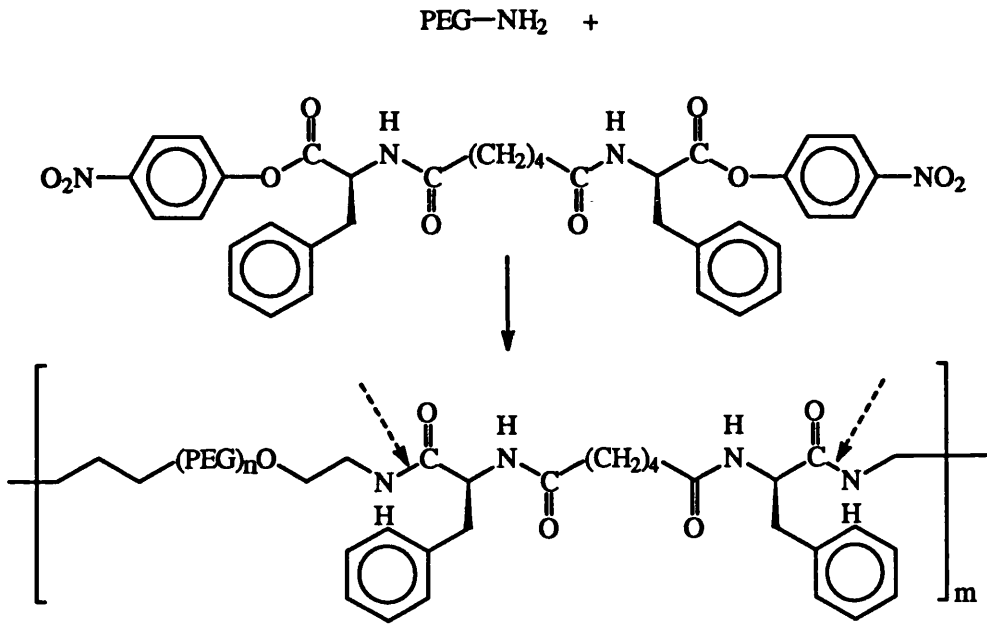
A synthetic method has been developed (292) for the iterative preparation of PEG oligomers. Although this process does not constitute polymerisation, the strategy does provide discrete PEG oligomers all of homologous size. The scheme involves alkylating the sodium anion of mono-tritylated ethyleneglycol oligomers with O-tosyl-O'-allyl-triethylene glycol or O-tosyl-O'-allyl-pentaethylene glycol (*Scheme 5.7*). Subsequent ozonolysis of the terminal olefin, followed by reduction with $NaBH_4$, provided the next higher mono-tritylated ethylene glycol oligomer which could be deprotected to the final glycol oligomer, or carried on in the iterative process.

PEG derivatives containing transformed terminal hydroxyls (NH_2, COOH, NCO groups) were treated with bifunctional reagents, [1] N, N'-adipoyl bis (phenylalanine *p*-nitrophenyl ester) (*Scheme 5.8*) or [2] N,N'-bis (phenylalanyl) hexamethylenediamine (*Scheme 5.9 and 5.10*) to generate a number of polymeric substrates.



Scheme 5.7.

PEG Polymers Derived from Bifunctional Reagents, *Scheme 5.8.*



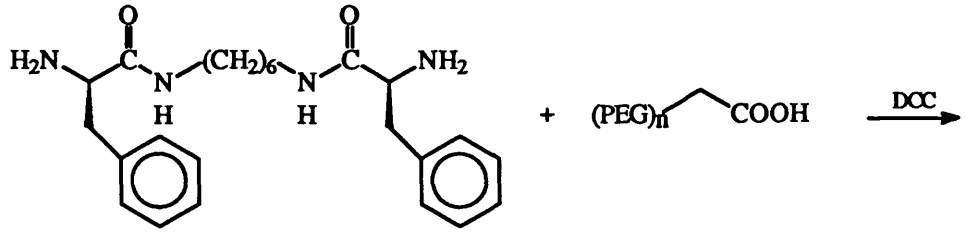
Scheme 5.8.

Denotes cleavage by:-

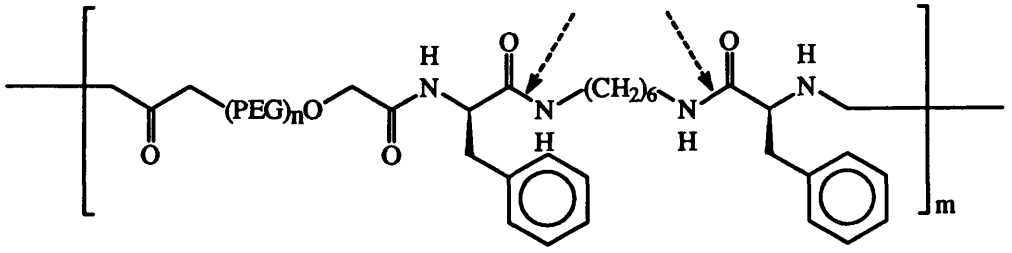
------► chemical hydrolysis.

——► chymotrypsin.

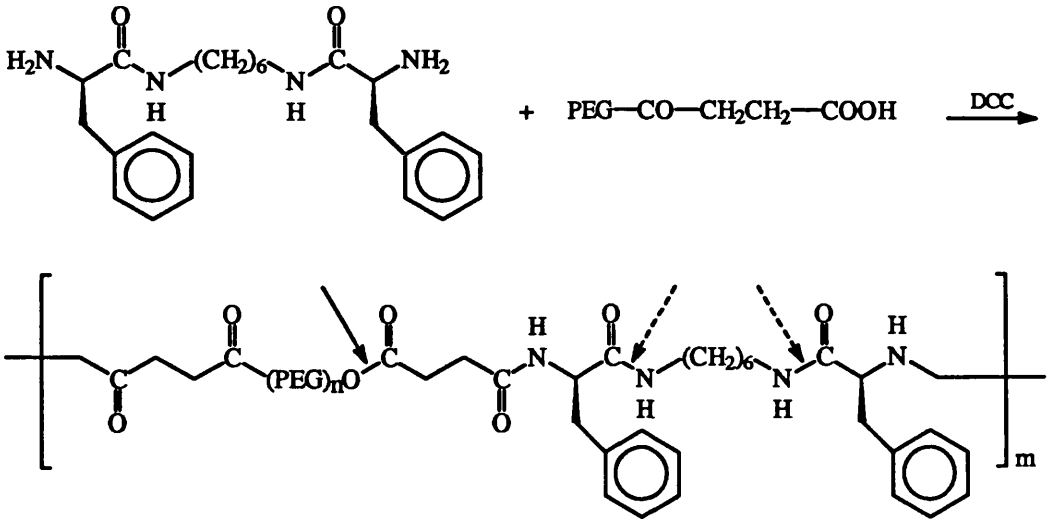
PEG Polymers Derived from Bifunctional Reagents, *Figure 5.9.*



Scheme 5.9.

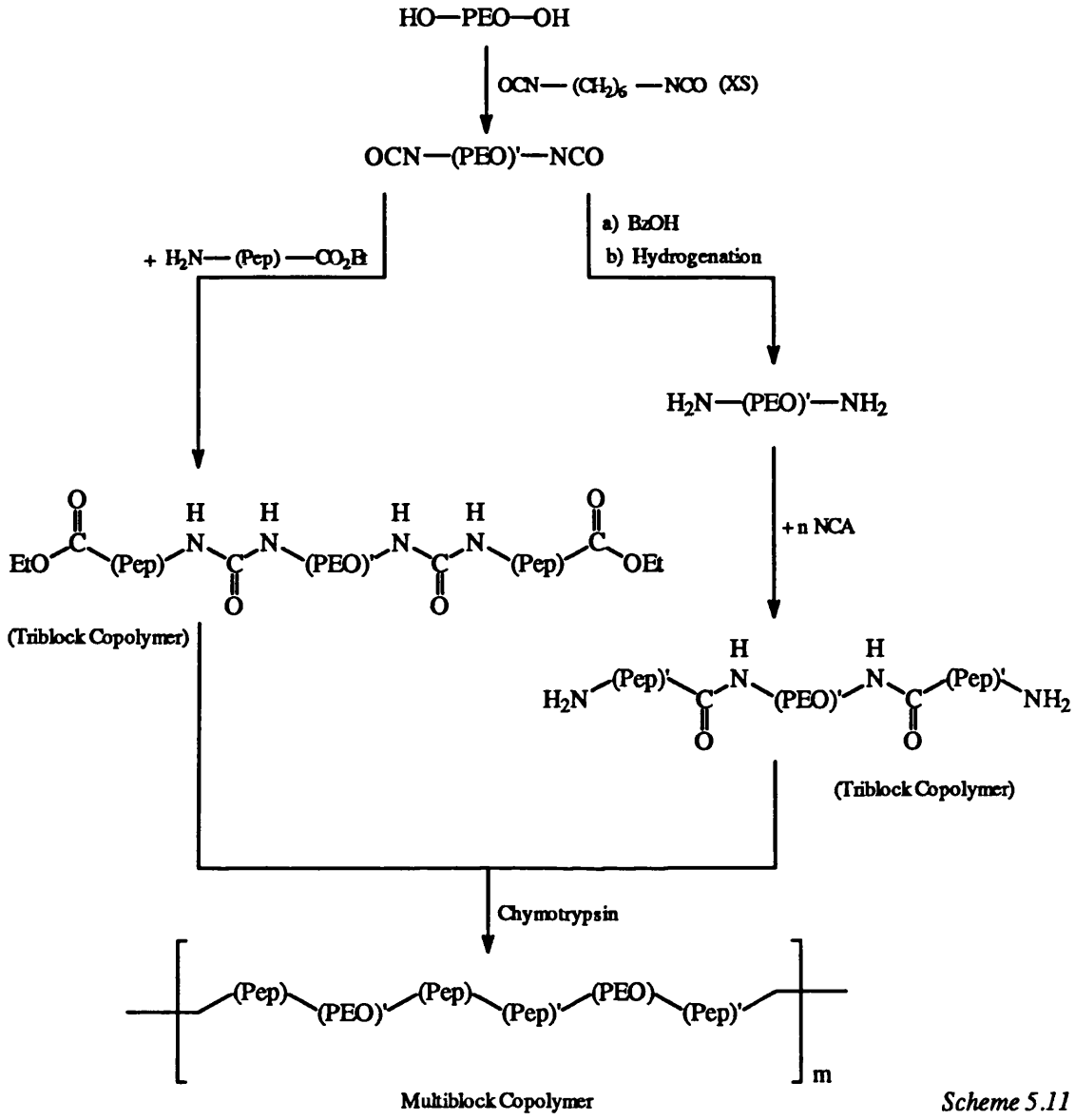


PEG Polymers Derived from Bifunctional Reagents, *Scheme 5.10.*



Scheme 5.10.

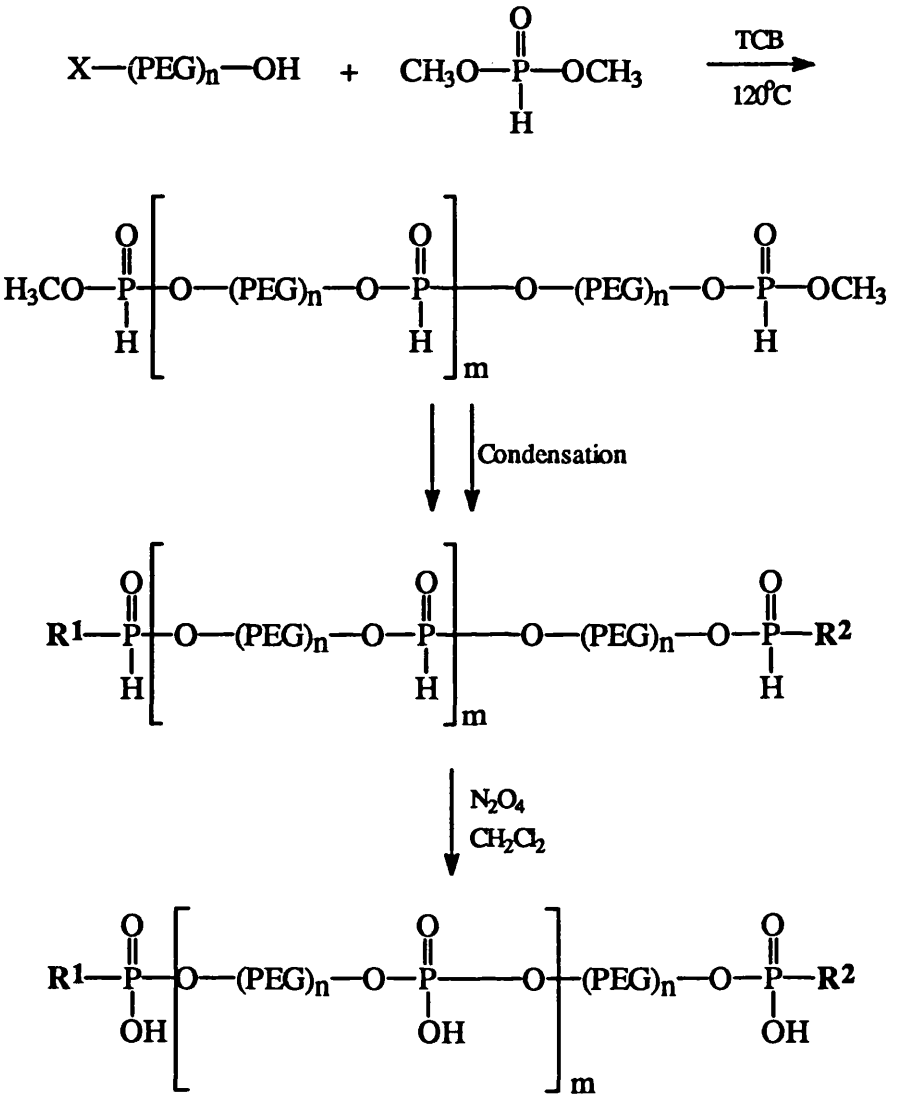
Oligopeptides linked to poly(ethylene oxide), PEO, undergo an enzymatically catalysed polycondensation to form high molecular weight segmented block copolymers reported Leonhardt *et al* in 1982 (319). PEO-isocyanate (PEO-NCO) was allowed to react with oligopeptides (Leu-Phe) which had free amino and ethyl ester end groups (*Scheme 5.11*). Triblock prepolymers with C-terminal peptide blocks were thus obtained. The PEO-NCO was treated with benzyl alcohol followed by cleavage of the Z-protecting group, which gave the required PEO with two amino acid end groups. The bis(amine) was used as the initiator for the polymerisation of phenylalanine N-carboxyanhydride. The enzymatic coupling of the two triblock copolymers formed a block copolymer under the action of chymotrypsin.



Scheme 5.11

Pretula *et al* (320) have phosporylated PEG (200) with dimethyl phosphite leading to the polyphosphites and the PEG-polyphosphates on oxidation, (see *Scheme 5.12*). Following condensation of the two end groups dimethyl phosphite was released together with the formation of the higher molecular weight polymer. The phosphonyl group contains a reactive P-H bond which can be used for the preparation of various derivatives, *e.g.* direct attachment of amino acids. Oxidation of PEG-200 polyphosphite led to the corresponding

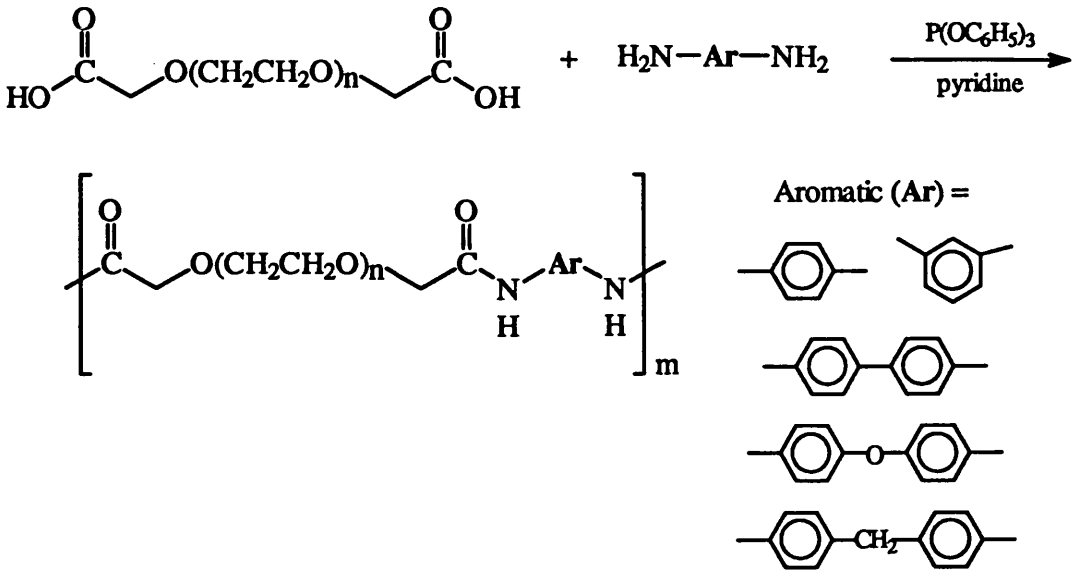
polyphosphate without decreasing the degree of polymerisation. Experiments at basic pH showed that this polymer slowly hydrolyses and may be useful as a biomaterial.



Scheme 5.12.

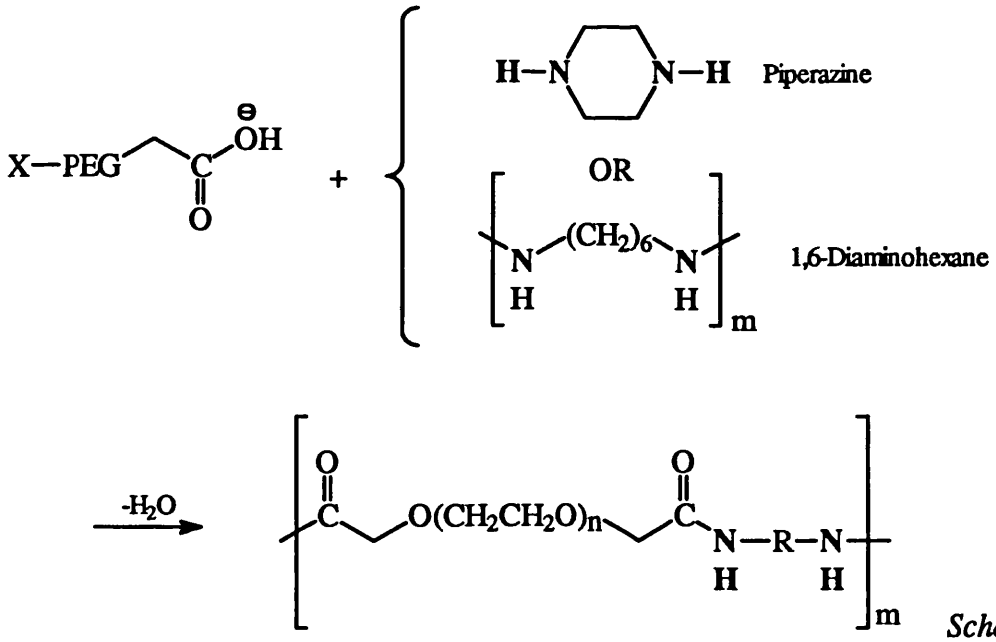
Polyoxyethylene (PEO) diacid with carboxyl groups in both terminal positions is suitable for the preparation of various condensation polymers. Recently Imai *et al* (321) reported a direct polycondensation prompted by triphenyl phosphite and pyridine in which

polyether-amides were readily obtained from aromatic diamines and PEO-diacids, (*Scheme 5.13*). The polyether amides are very hygroscopic and readily soluble in water and a wide range of other solvents including benzene, acetone and methanol.



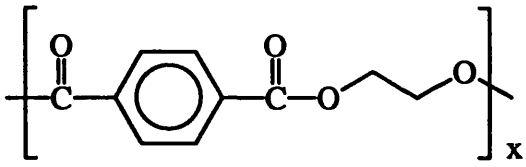
Scheme 5.13.

Further polymerisation reactions based on PEO-diacids involve the copolymerisation of piperazine and 1,6-diaminohexane salts (322). These salts are analogous to the nylon 6,6 salt formed between adipic acid and diamino hexane. They, like the "nylon salt" can be converted to the high molecular weight through the removal of water (see *Scheme 5.14*).



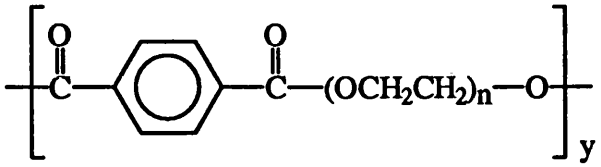
Scheme 5.14.

A new type of polyether-polyester block copolymer consisting of two components of polyethers and one polyester has been synthesised by Wang (323). A mixture of polytetramethylene glycol (PTMG), PEG, dimethyl terephthalate (DMT), ethylene glycol (EG) and tetrabutyl titanate catalyst (TNBT) were made to undergo an ester exchange reaction. A mixed polyether-polyester block copolymer was obtained and tested for it's biomedical properties. By altering the proportion of the mixed polyether content the different hydrophilicities and hydrophobicities of the copolymer were investigated (*Scheme 5.15*).



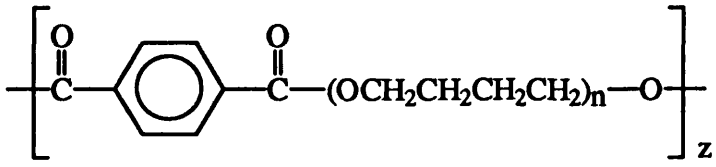
Polyester; (1)

PET



Polyether + (1)

PEGT



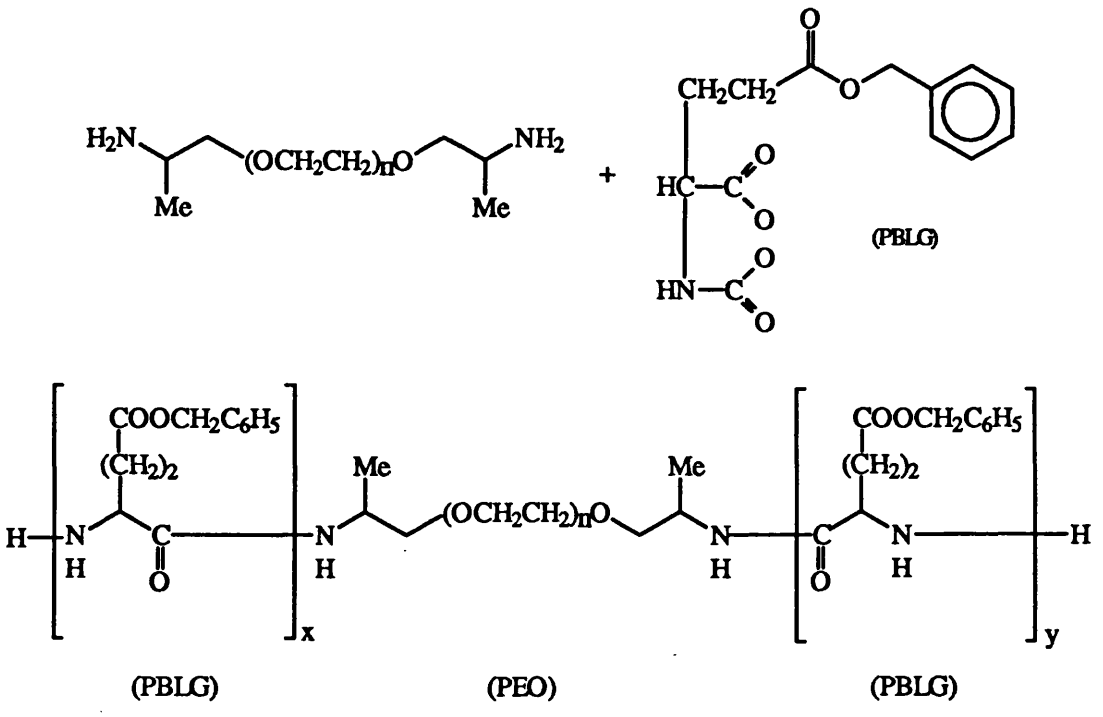
Polyether + (1)

PTMGT

Scheme 5.15.

The copolymers showed an improved biocompatability and anti-thrombogenicity, through the introduction of hydrophilic PEG monomers into the polymer chain.

ABA-type block copolymers composed of poly(γ-benzyl L-glutamate) (PBLG) and PEO were synthesised by polymerisation of γ-benzyl L-glutamate N-carboxyanhydride, initiated by primary amines at the terminal ends of the PEO chain (335), (*Scheme 5.16*). The ABA type block copolymer of incompatible A and B polymer chains is of interest with special reference to their biocompatability or antithrombogeneicity. The PBLG / PEO / PBLG triblock copolymers are promising polymers for medical use.



Scheme 5.16.

5.4. The PEG Hydrogels.

One approach to hydrogel formation involves the use of Pluronic F-127, a block copolymer of hydroxyl terminated propylene and ethylene oxides. At low temperatures a 20% solution of Pluronic is clear, non-toxic and liquid. This solution can be poured easily onto a burn site and at body temperature will form a clear gel which fills the crevices in the wound (324).

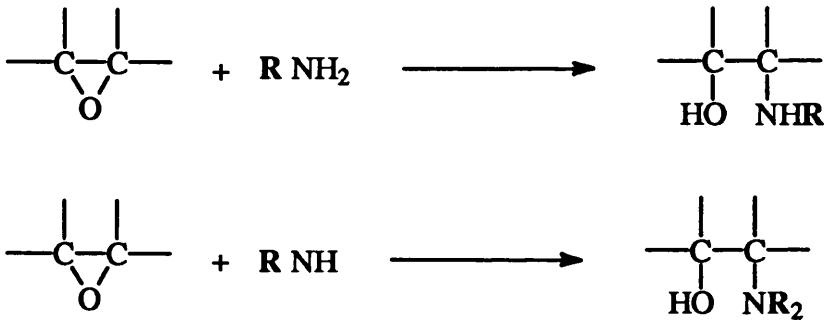
One of the earliest and most widely studied hydrogel based wound dressing is the Hydron Burn Bandage. The dressing is formed directly on the burn from a two compartment system, poly (2-hydroxyethylmethacrylate) [*p*(HEMA)] and polyethylene glycol as the solvent. Alternate layers of PEG and *p*(HEMA) are applied to the wound until three or four layers have been built up. The PEG dissolves the *p*(HEMA) forming a

saturated solution which solidifies after 30 mins. Further *in vivo* and *in vitro* studies in rats have confirmed the suitability of the dressing for the delivery of antimicrobial agents. Similarly clinical trials have shown that the dressing will release a variety of topical agents, *e.g.* gentamicin, silver nitrate and nitrofurazone.

5.5. Discussion.

5.5.1. Analogues to the polymerisation process.

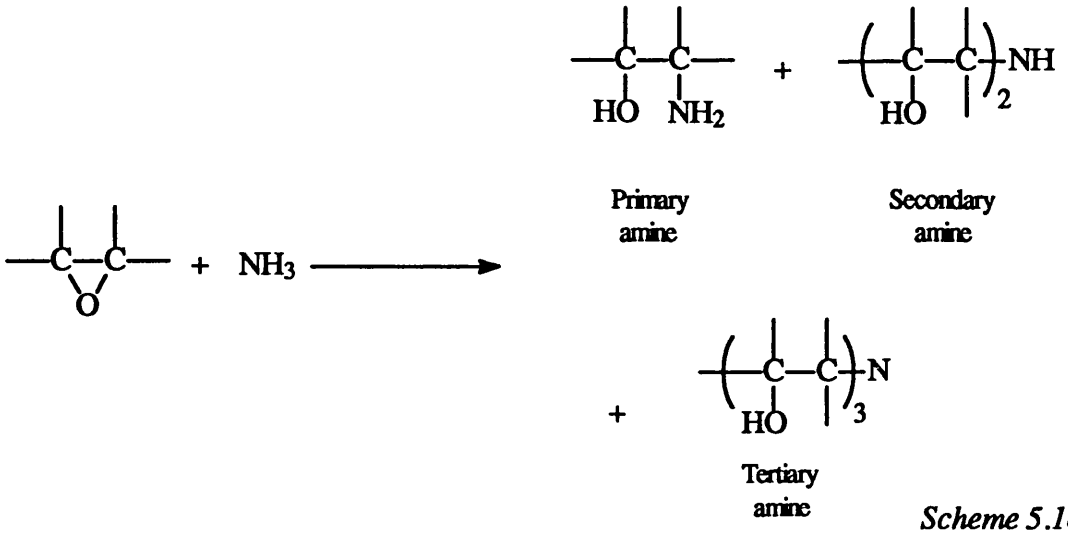
The amination of the epoxides with, for instance a PEG-primary amine, *e.g.* PEG ethylene diamine or a PEG-secondary amine, *e.g.* PEG N-methyl-2-methoxyethylamine would afford the secondary and tertiary amines, respectively, (*Scheme 5.17*).



Scheme 5.17.

Both strategies were used in the polymerisation studies involved in this project.

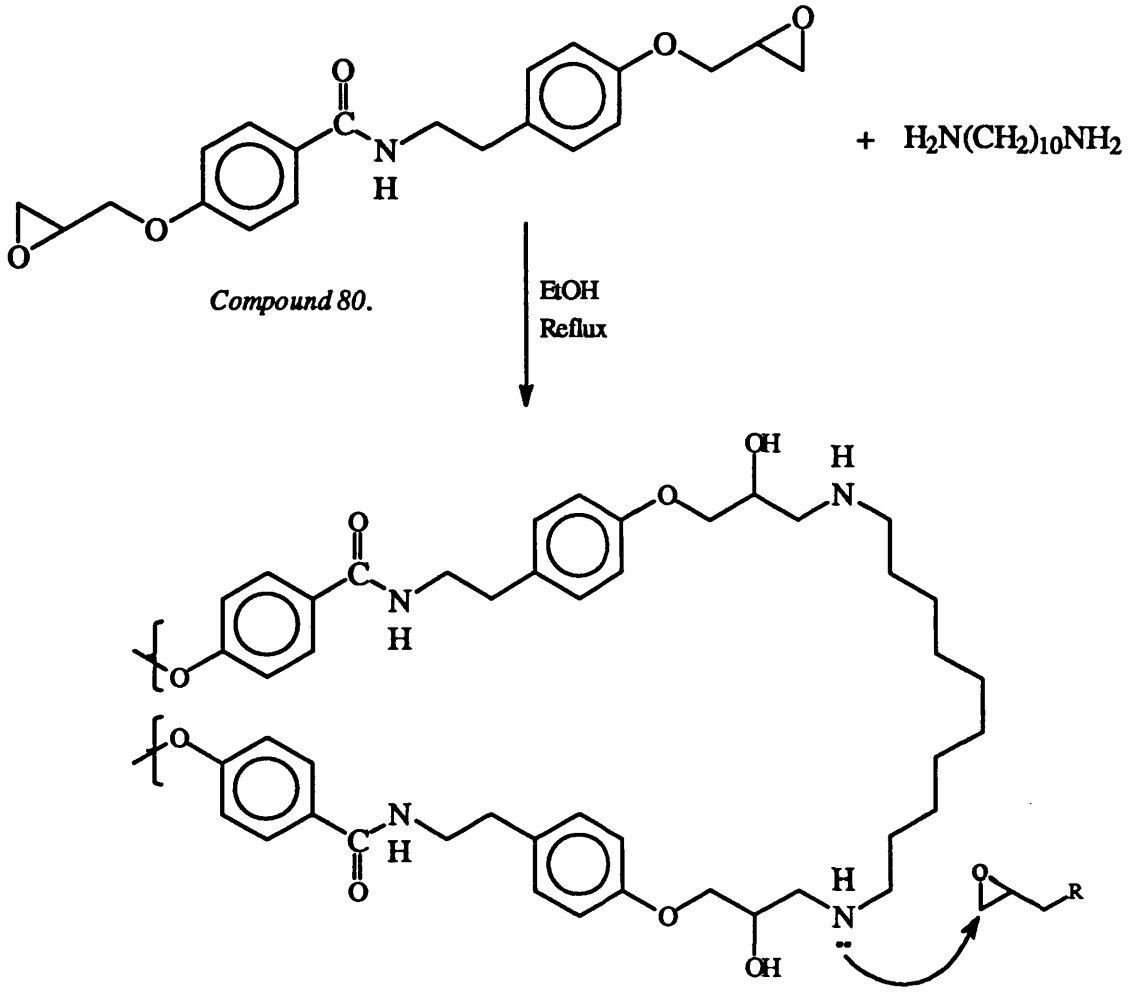
The reaction between epoxides and ammonia is a general and useful method for the preparation of β-hydroxyamines. Ammonia gives largely the primary amine but also some secondary and tertiary amines, (*Scheme 5.18*).



Scheme 5.18.

In order to test the feasibility of a reaction between a bis(glycidyl)ether and a primary amine a protocol was initiated using analogues.

Iterative addition was shown to occur when 4-(oxiranylmethoxy)-N-(2-(4-oxiranyl-methoxy)phenyl)ethyl)benzamide (*Compound 80*) was treated with decane-1,10-diamine (*Scheme 5.19*) in place of the activated PEG. The bis(glycidyl) ether was added slowly to the bis amine in equimolar concentrations and the reaction was boiled in ethanol for 36 hours. A product (*Compound 72*) was isolated and the data from mass spectroscopy (FAB [+]) clearly showed a mother ion peak (*m/z* 542) followed by iterative peaks (*m/z* 1083) indicating that the reaction had been successful to some degree. Unfortunately the M.S. range was not sufficiently wide to be able to identify other fractions other than the monomer and dimer. It was unlikely that the secondary amine produced on the product was capable of playing any further significant role in the reaction. However, if the secondary amine was to take part in an additional reaction, with an electrophile group, then the linear nature of the polymerisation process would be lost and cross-linking would result.



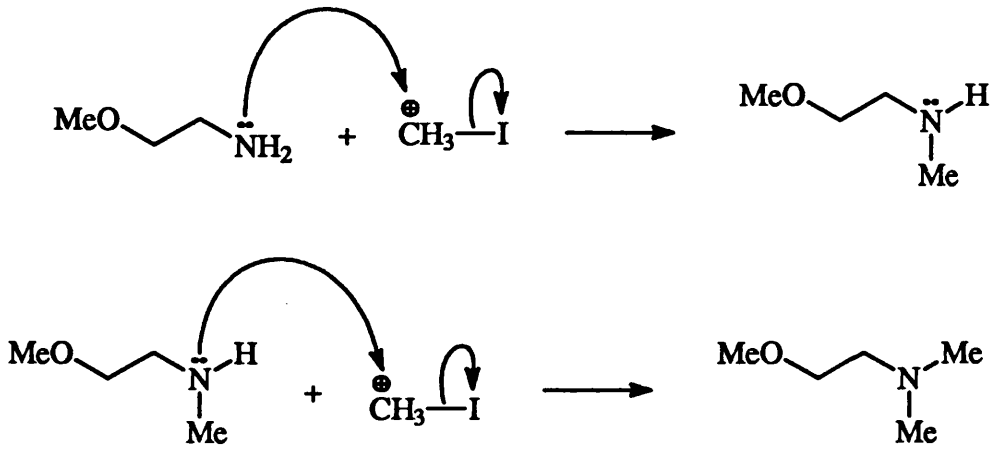
Compound 80.

Compound 72.

Scheme 5.19.

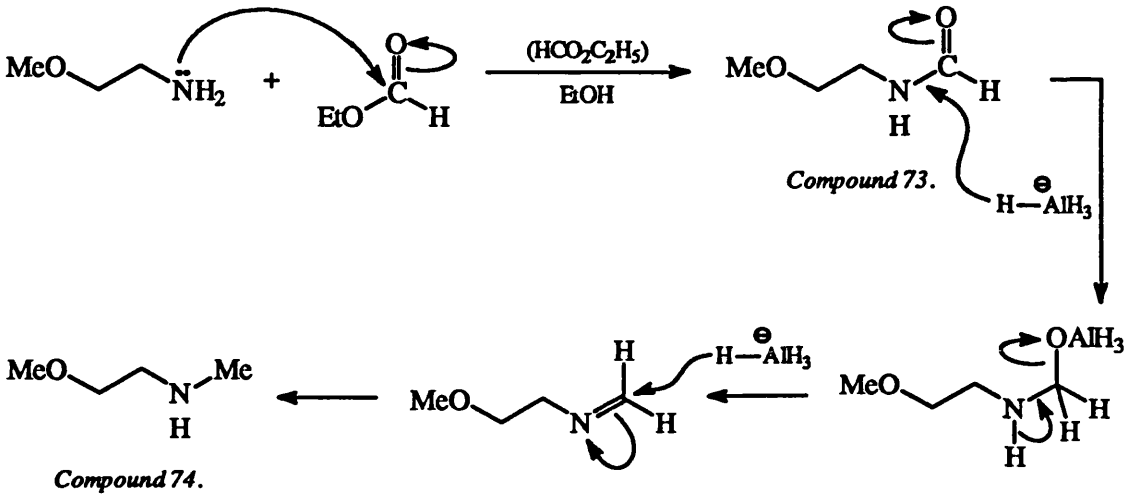
The problem of crosslinking at the primary amine site and characterising the resultant polymer was solved to a large degree by incorporating a monofunctional secondary amine into the polymerisation process as a model. N-Methyl-2-methoxyethylamine was allowed to react with the bis(glycidylethers) to determine the feasibility of the reaction at the methyl substituted secondary amine site. Iterative chain extension was made impossible due to the prescence of a terminal methoxy protecting group on the analogue compound. By incorporating this small, simple analogue into the reaction, the product, unlike the polymer could be characterised fully by standard laboratory techniques.

N-Methyl-2-methoxyethylamine can be made in a number of ways. One straightforward strategy involves the use of iodomethane in an electrophilic substitution reaction with 2-methoxyethylamine, (see *Scheme 5.20*). A disadvantage with this approach is that the iodomethane is an aggressive electrophile and is capable of substituting the newly formed secondary amine further to produce a tertiary amine with subsequent quaternisation. This would prevent any reaction at all with the oxiranyl groups.



Scheme 5.20.

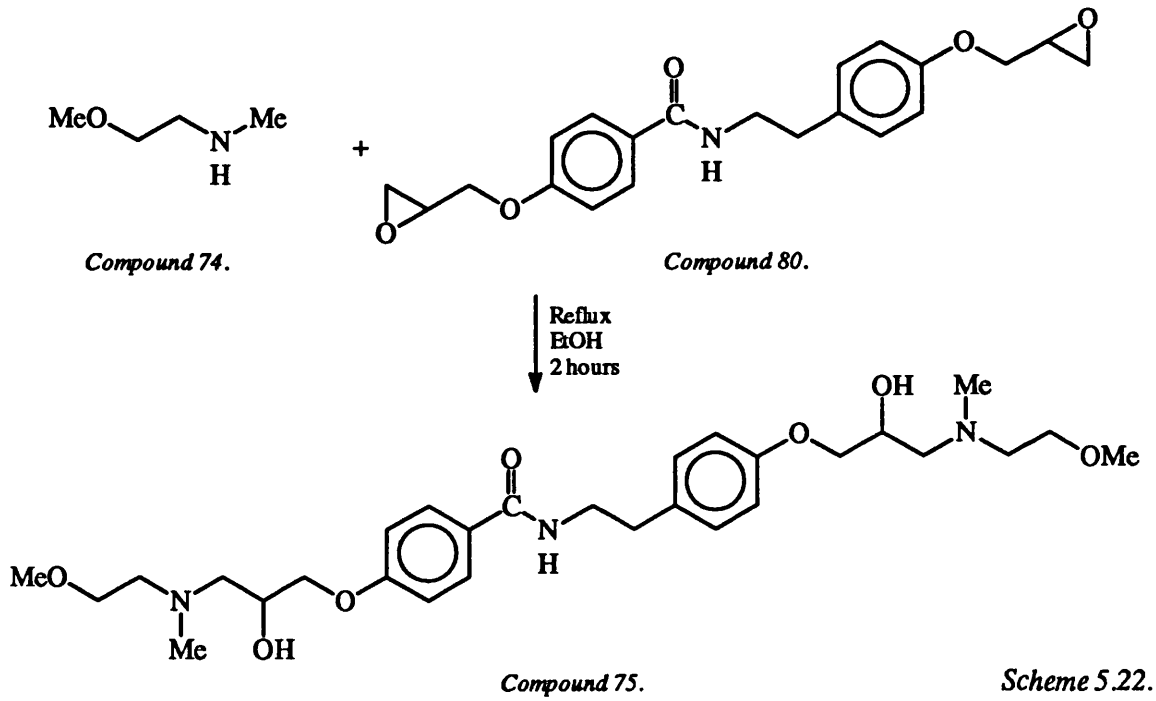
An alternative route, which was adopted, was to convert 2-methoxyethylamine to the formamide using an excess ethyl formate. The conversion to N-2-(methoxyethyl)formamide was completed when the excess ethyl formate, which served to introduce the formyl group was removed by distillation and a product (*Compound 73*) was afforded in good yield. This material was treated with lithium aluminium hydride ($LiAlH_4$) in THF, where the formamide was reduced to afford a product which was isolated from any impurities by distillation at 95° C. The known compound N-methyl-2-methoxy ethylamine, (325) (*Scheme 5.21*) was isolated in poor yield and characterised as the methylamino compound, (*Compound 74*) by proton spectroscopy which corroborated the identity of this compound with observation of the newly formed methyl singlet at δ 2.45.



Compound 74.

Scheme 5.21.

As a model reaction, to test the feasibility of polymerisation with a secondary amine, a simple bis (oxiranyl) (*Compound 80*) [4-(oxiranylmethoxy) N-(2-(4-oxiranylmethoxy)-phenyl)ethyl)benzamide] was allowed to react with the methyl protected amine, (*Scheme 5.22*).

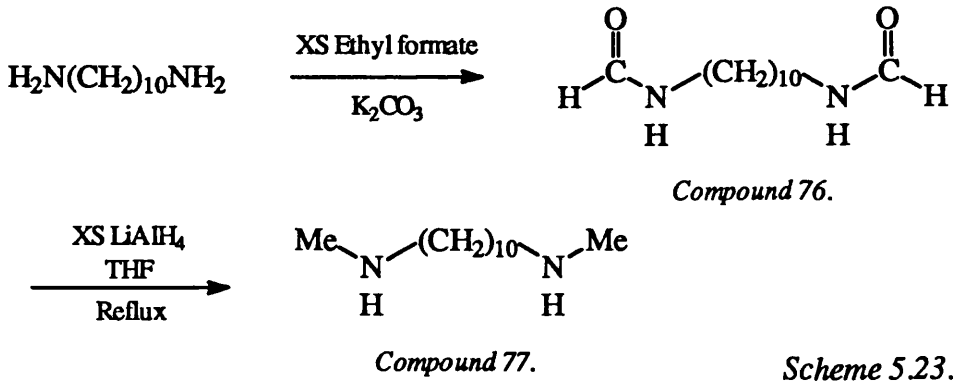


Compound 74. *Compound 80.*

Compound 75. *Scheme 5.22.*

A material was isolated from this reaction in good yield and characterised further with proton spectroscopy. Comparison of the integrals of the N-methyl singlets at δ 2.23 and δ 2.24, of the methoxy signals at δ 3.19 and δ 3.20 and of the aromatic cluster between δ 6.85 - 7.78 corroborated the identity of the material as *Compound 75*. This characterisation was confirmed by observation of the M + H ion at *m/z* 548 in the mass spectrum.

The preliminary polymerisation experiments using decane-1,10-diamine were re-assessed in the light of the successful results obtained from the secondary amine analogues. The PEG analogue decane-1,10-diamine was converted to the formamide (*Compound 76*), again using an excess of ethyl formate and reduced to the methyl compound with $LiAlH_4$ to give N,N'-dimethyldecane-1,10-diamine, (*Compound 77*), (*Scheme 5.23*). This material was identified by ^1H NMR and mass spectroscopy with the observation of an ion peak at M + H *m/z* 201 and *m/z* 199 for FAB [+] and FAB [-] respectively.



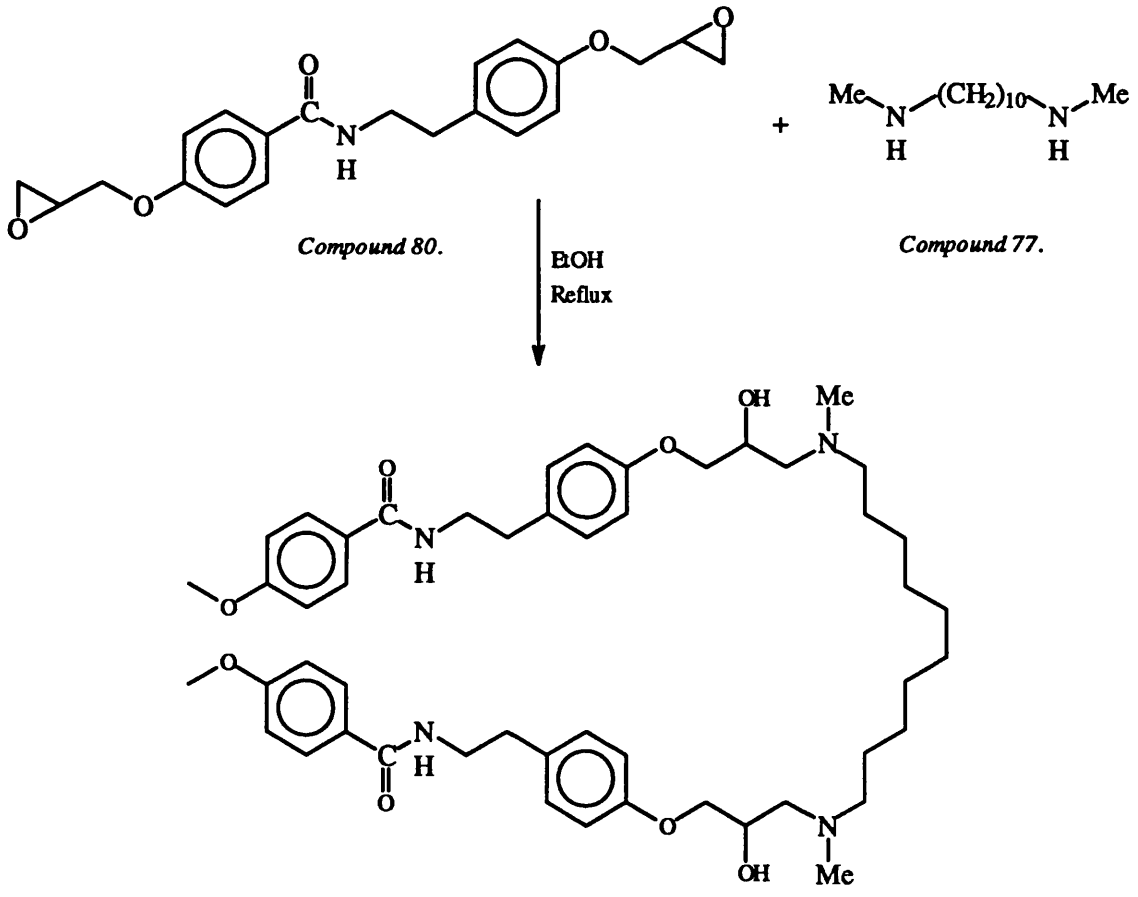
Compound 76.

Compound 77.

Scheme 5.23.

The formation of the secondary amine, N,N'-dimethyldecane-1,10-diamine which was capable of taking part in a polymerisation reaction was an important step. It was hypothesised that the reaction of the bis amine with the bis(glycidylether) would form a product with a tertiary amine, incapable of any further reaction with an electrophile and crosslinking would be avoided, (see earlier model reaction scheme, *Scheme 5.17*).

In view of this a mixture of *Compound 80* and N,N'-dimethyldecane-1,10-diamine was refluxed for 16 hours in ethanol and allowed to cool, (*Scheme 5.24*). The product (*Compound 78*) was separated into two populations each with different solubility's. It was envisaged that the high molecular weight polymeric products had a low solubility whereas the smaller oligomers remained in solution. The ethanol was removed by evaporation and these low molecular weight products were obtained as an oil in moderate yield. By incorporating a secondary amine onto the PEG the danger of cross linking through multiple reactions at the nucleophilic site was abolished and the linear nature of the polymer was conserved.



Compound 80. *Compound 77.*

Compound 78.

Scheme 5.24.

Chapter 6. Conclusion

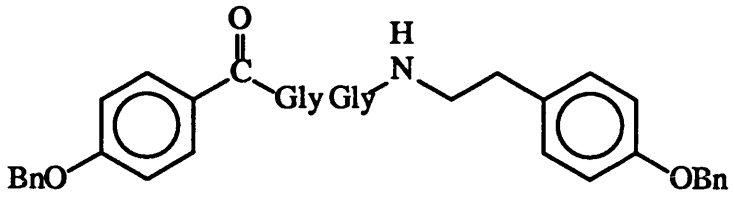
6.1. Conclusions.

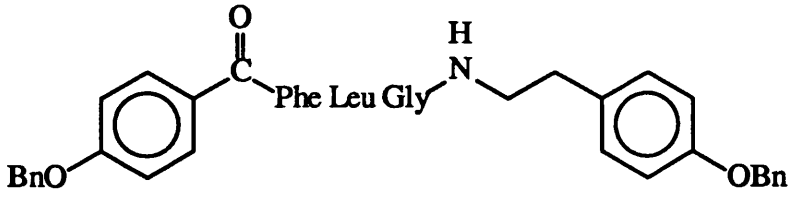
A study was undertaken to investigate a series of copolymers based on poly(ethylene glycol) [PEG] and a biodegradable oligopeptide spacer for use as polymeric prodrugs. The preparation of a number of oligopeptides bearing protected phenolic side groups, suitable for further derivatisation has been demonstrated by solution methods.

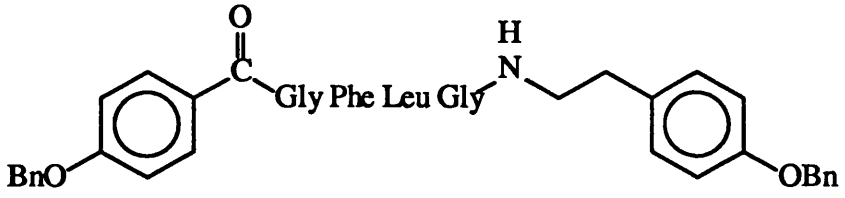
It was proposed that by generating a library of peptide sequences the enzymatic degradation of the spacer could be optimised. This would depend on the relative ease with which the polymeric substrates formed enzyme substrate complexes and therefore a series of up to five amino acids varying both in length and detailed structure were synthesised.

The polymerisation strategy adopted towards the alternating PEG-peptide linear polymer involved the reaction of α,ω-bis(glycidylether) peptides with α,ω-bis(methylamino) PEGs. To this end the oligopeptide chains were modified with a number of α,ω-side groups including the alkenes, bis (4-methoxyphenyl) and bis 4(benzyloxy)phenyl. Each was capable of further derivatisation to form the glycidyl ether, but the two former reagents presented epoxidation and cleavage problems respectively. The bis 4(benzyloxy)phenyl moiety provided a protecting group which could be cleaved readily in a hydrogenation step and was orthogonal to the requirements of peptide coupling.

Target Oligopeptide Intermediates Bearing bis 4-(benzyloxy)phenyl Protecting Groups.

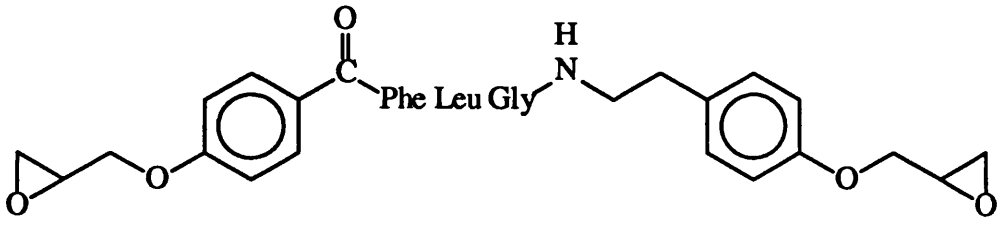






Following the conversion of the phenolic hydroxy groups to the bis(glycidylethers) by treatment with epichlorohydrin, these compounds were successfully allowed to react with primary and secondary amines in a model polymerisation reaction.

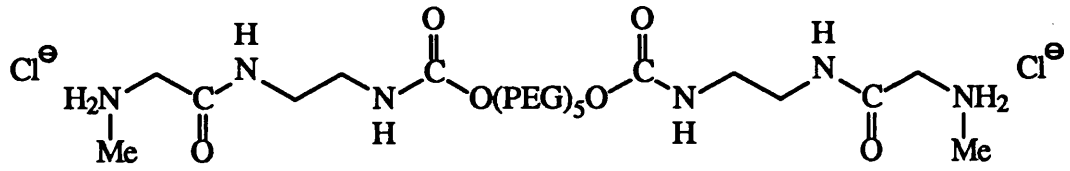
A Precursor to Polymerisation:- The Tripeptide PheLeuGly Modified With Glycidylether Groups.



The feasibility of treating α,ω-bis(glycidylether)peptides with a PEG-amine derivative to form a copolymer was addressed using penta(ethyleneglycol). The

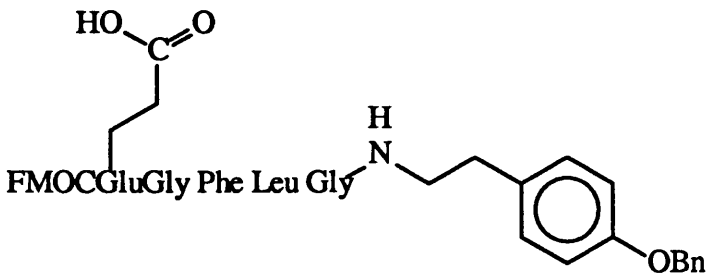
appropriately derivatised PEG monomers were provided in a straightforward conversion of the PEG diol to the α,ω-bis(chloroformate) with phosgene. Further treatment with BOCsarcosine N-(2-aminoethyl)amide gave the corresponding bis(urethane) and deprotection with hydrogen chloride gave the required α,ω-bis(methylamino) derivatised PEG. By introducing a secondary amine onto the PEG the risk of polymeric crosslinking was reduced.

Poly(ethyleneglycol) Derivatised With a Secondary Amine.



The potential to functionalise the PEG-containing polymers further has been investigated successfully by the inclusion of glutamic acid within the oligopeptide sequence. Appropriate protection of the α-amino and γ-carboxy groups of the glutamate led to the formation of the pentapeptide sequence which bears a side chain "handle".

Glutamic Acid Provides a Suitable Side Chain for Further Activation.



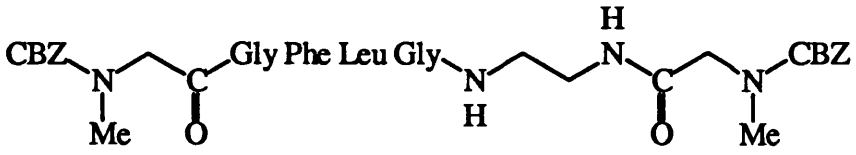
6.2. Assessments and Future Prospects.

The feasibility of treating an α,ω-bis(glycidyl)peptide with a methylamino group has been confirmed but tests using α,ω-bis(methylamino)PEGs are still pending. It is envisaged that an alteration in the molecular weight, *ergo* length of the PEG monomers used will generate a series of tailor made copolymers that will contain peptidic spacers at pre-determined intervals along the polymer backbone. However, to achieve any uniformity at all would entail limiting the molecular weight distribution to a narrow range. This may be problematic with some of the large molecular weight PEG compounds.

Interest should be focused on the ability of various PEG monomers to influence the solubility of the oligopeptide conjugate. In addition the degree of polymerisation will dictate the molecular weight and consequently the hydrodynamic radius of the final copolymer. Such issues have important ramifications if *in vivo* studies are to be considered. A target mass of between 80 - 120 KDa should be considered if rapid renal clearance (>20 KDa) is to be avoided and extravascular migration made feasible.

A comprehensive series of enzymatic degradation studies on future PEG / peptide copolymers are awaited with impatience. It is hypothesised that the linear PEG chains should not be the cause of any serious steric hindrance to the formation of the enzyme substrate complex. However, the presence of the aryl side groups adjacent to the oligopeptide may interfere with the S' - P interactions to reduce the overall lability of the sequence. This problem could be addressed in the development of a second generation of protected peptide segments whereby the aryl groups are replaced with CBZsarcosine N-(2-(aminoethyl)amides. This approach is outlined below.

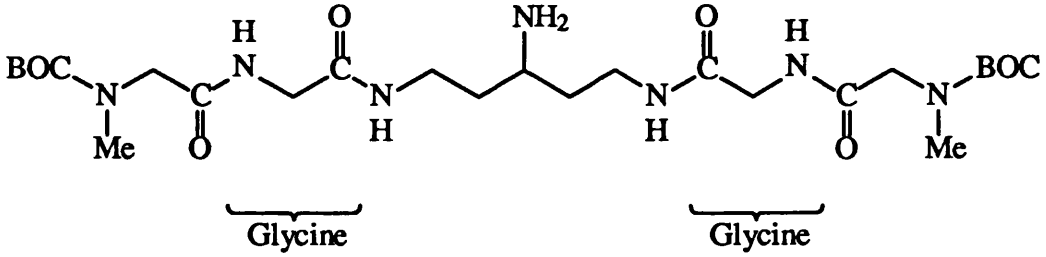
A Precursor to the Second Generation of PEG / Peptide Copolymers.



In contrast to those compounds discussed in the previous chapters, copolymerisation of these latter compounds is achieved *via* the reaction with α,ω-(glycidyl)PEGs.

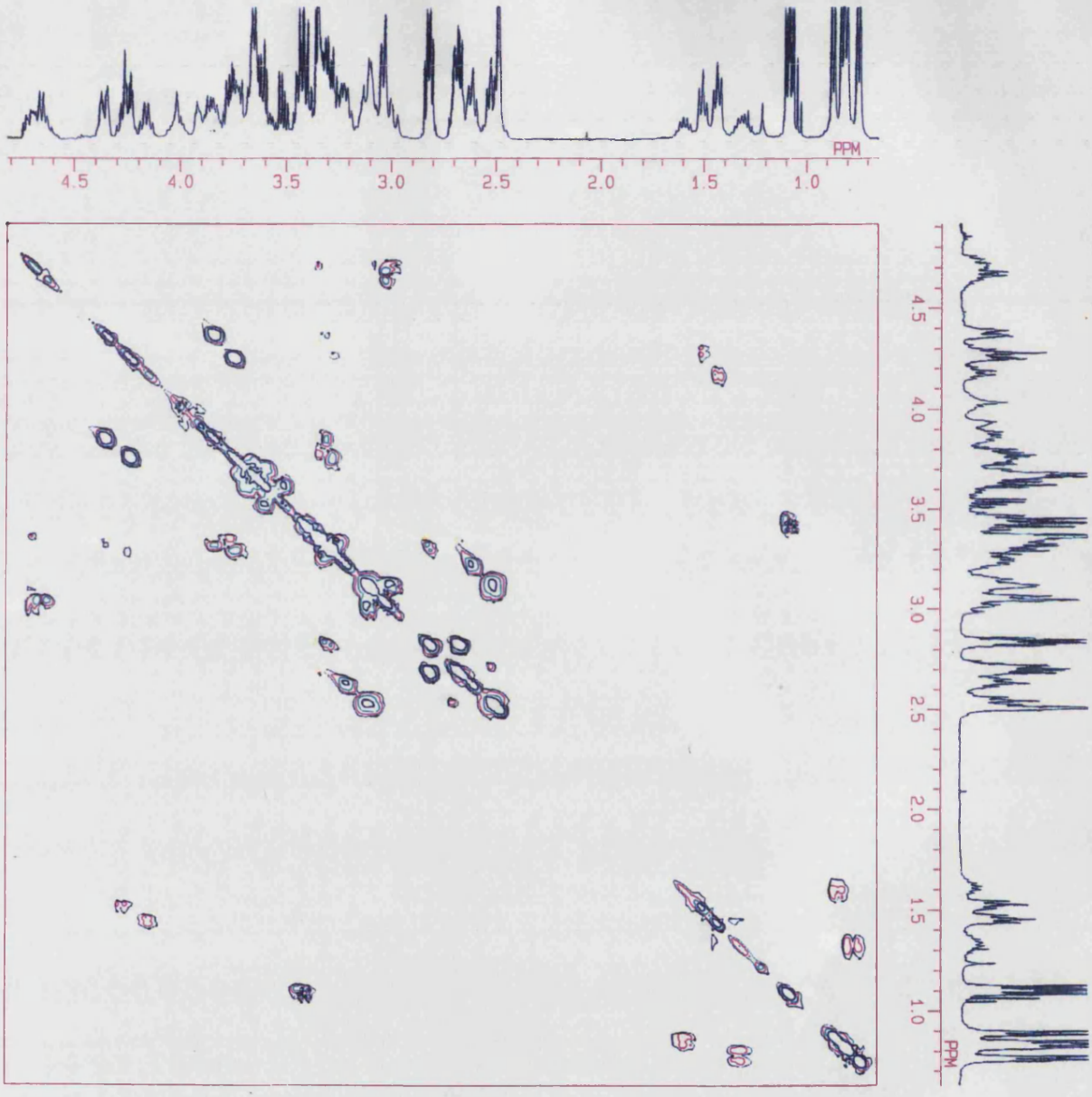
A variation on the above procedure could be found in a third generation of modified oligopeptides. In this strategy two BOCsarcosine protected amino acids are linked with a spacer *via* the peptide α-carboxy terminals. This approach is outlined below, using glycine as an example. These analogues are simple, accessible to enzymatic degradation and can be derivatised further through side chain activation. The symmetry of this peptide unit and the symmetry of the PEG unit obviate any potential problem of random orientations of "directional" peptides in the polymers.

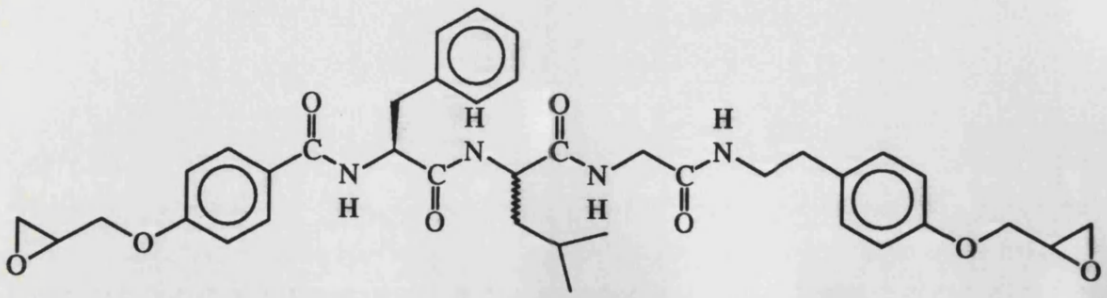
A Spacer Unit Separates Two Glycines in the Third Generation of Compounds



More exploration in the area of soluble polymeric precursors is warranted as a viable alternative to the present day drug delivery regimes. Furthermore it is expected that the future development of copolymers based on PEG and biodegradable oligopeptide sequences will have a seismic effect on the whole of the drug targeting landscape.

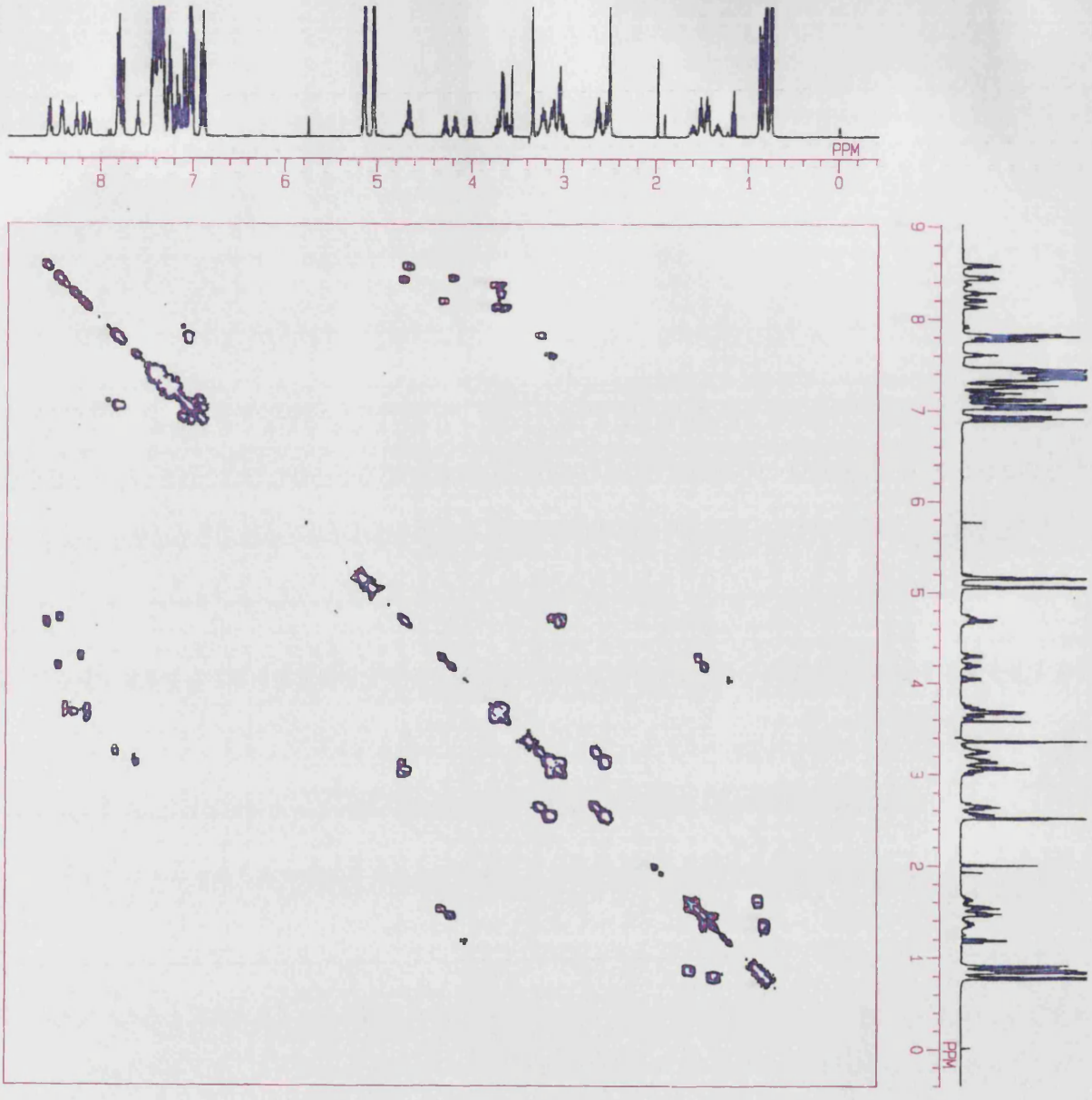
Appendix 1. COSY Spectrum for Compound 79.

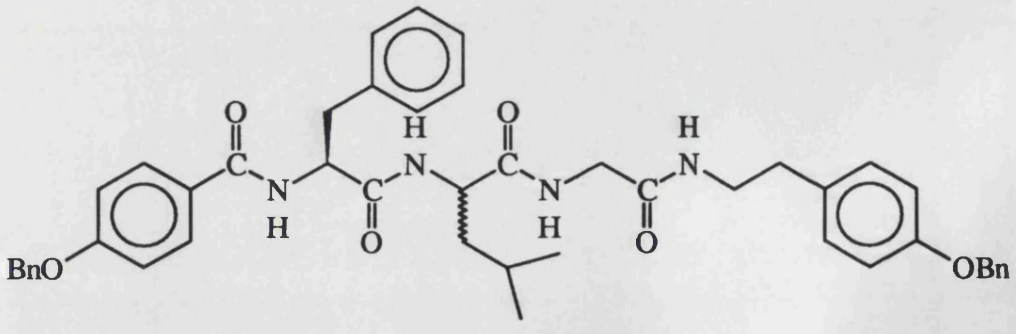




Compound 79.

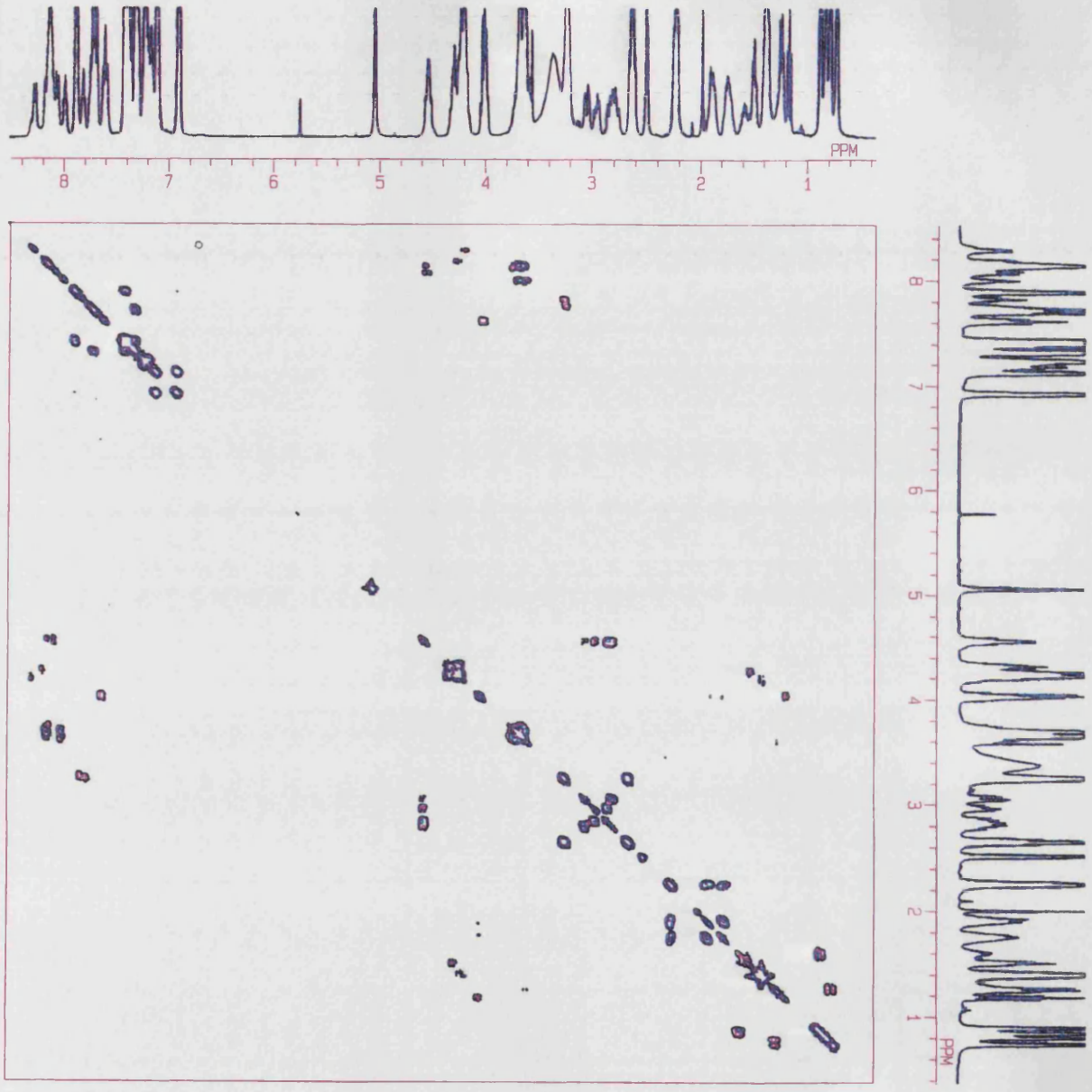
Appendix 2. COSY Spectrum for Compound 22.

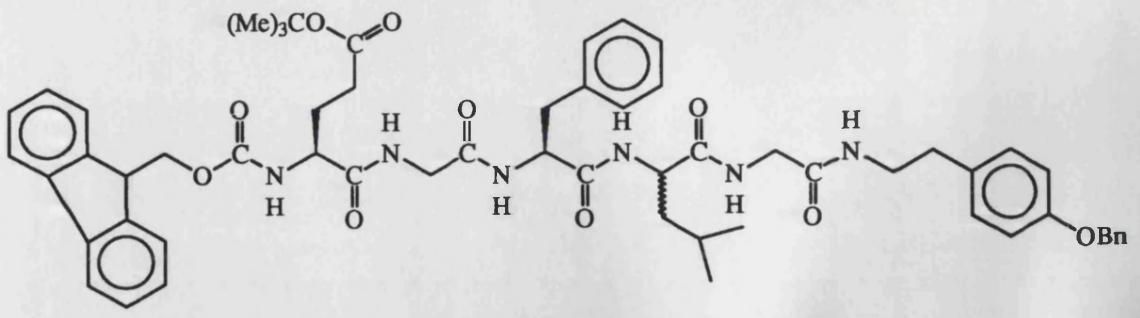




Compound 22.

Appendix 3. COSY Spectrum for Compound 62.





Compound 62.

Experimental.

Abbreviations.

Anal.	Analysis.
Aq.	Aqueous.
BOC	1,1-Dimethylethoxycarbonyl.
Bn	Benzyl.
CBZ	Phenylmethoxy carbonyl.
C.I.	Chemical ionisation.
DCC	1,3-Dicyclohexylcarbodiimide.
DMF	Dimethylformamide.
DMAP	4-(Dimethylamino)pyridine.
E.I.	Electron impact.
Et_2O	Diethyl ether.
EtOAc	Ethyl acetate.
EtOH	Ethanol.
F.A.B.	Fast atom bombardment.
FMOC	9-Fluoroenylmethoxy carbonyl.
h	Hour.
HOBt	1-Hydroxybenzotriazole.
HONSu	N-Hydroxysuccininmide.
H_2SO_4	Sulphuric acid.
MeCN	Acetonitrile.
min.	Minutes.
MCPBA	3-Chloroperoxybenzoic acid.
NMR	Nuclear magnetic resonance.
Pd / C	Palladium on activated carbon (10%).
PFP	Pentafluorophenyl.
PMA	Phosphomolybdic acid.
TCP	2, 4, 5-Trichlorophenyl

General Information.

Infra-red (I.r.) spectra were recorded on a Perkin-Elmer 782 spectrometer, using KBr discs.

^{1}H, ^{13}C and ^{19}F N.M.R. spectra were recorded usimg a Joel JNM-GX270 or with a Joel JNM-EX 400 spectrometer. Samples were dissolvedl in $CDCl_3$ or $(CD_3)_2SO$ and chemical shifts (δ) were measured in ppm relative to tetramethylsilane (TMS) which was used as an internal standard. Values of the number of protons for each signal are given between the brackets. ^{19}F-NMR was measured against $CFCl_3$ internal standard. Multiplicities are indicated as follows; s (singlet); d (doublet); dd (double doublet); t (triplet); dt (double triplet); ddt (double double triplet); q (quartet) and m (multiplet).

Mass spectra were obtained using a VG 7070E analytical mass spectrometer, University of Bath, and reported in the form *m/z* (intensity relative to base = 100) for selected ions. Spectra were obtained from electron impact (E.I.), chemical impact (C.I.) or fast atom bombardment (FAB) ionisation techniques. Accurate mass spectra were obtained from the EPSRC Mass Spectrometry Service, University of Wales, Swansea having been run on a VG-Autospec Instrument using two known internal standard reference ions from polyethylene glycol to straddle the unknown mass.

Elemental analysis was carried out using a EMA System 1106, microanalysis unit, University of Bath.

Where appropriate, the stereochemistry of the compounds containing one or more chiral centres was investigated. Spectra generated by NMR revealed the presence of diastereo-isomers which are assigned in the data using isomer 1 or isomer 2. Measurements of optical activity were carried out using the Optical Activity Ltd. AA-10 polarimeter.

All reactions were monitored by thin layer chromatography (T.L.C.) which was performed on pre-coated silica sheets manufactured by Merck; (Merck t.l.c aluminium sheets silica 60 F_{254}, Art. no. 5635). Products were visualised in a variety of ways; ultraviolet (U.V.) light; spraying with phosphomolybdic acid in methanol followed by heating; iodine vapour; iron III chloride in methanol; 4-methoxybenzaldehyde, ninhydrin in methanol. The following solvent systems were used: (A): chloroform; methanol (18: 1); (B): dichloromethane; (C): ethyl acetate, (D): 5% MeOH / EtOAc. Chromatographic purification was carried out by forced air chromatography using Sorbsil C60 (0.040 - 0.063 mm) silica gel in the noted solvent

system. Suspensions where appropriate were filtered through a filter agent, Celite ® 521; (John Manville Corp.).

Melting points (corrected) were determined using a Thermo Galen Kofler block with samples between two glass plates.

Chemicals were purchased from Aldrich, Sigma, Novabiochem and Fluka as reagent grade and used without further purification. Licences for use of controlled substances were secured from the Home office where appropriate.

All solvents were evaporated under reduced pressure. When required tetrahydrofuran was passed down an alumina column and distilled from sodium wire and benzophenone. Ethanol was dried over magnesium turnings and iodine and distilled. Dimethylformamide, dichloromethane and triethylamine were dried over sodium hydride, distilled and stored over 4 Å molecular sieves. Light petroleum refers to the fraction with boiling point 60 - 80°C. Organic phases were dried over $MgSO_4$ unless otherwise stated.

Compound 1. N-(1,1-Dimethylethoxycarbonyl)phenylalanine. Phenylalanine (2.00 g, 12.10 mmol) was dissolved in water (23 mL) containing NaOH (0.48 g, 12.10 mmol) and stirred vigorously for 12h with a solution of di-t-butyl dicarbonate (3.96 g, 18.15 mmol) in 1,4-dioxan (10 mL). The reaction was quenched with diethyl ether and the aq. fraction was acidified with H_2SO_4 (10%) and extracted with EtOAc. The solvent was dried and evaporated to afford N-(1,1-dimethylethoxycarbonyl)phenylalanine (3.14 g, 98%) as white crystals: mp 79 - 80°C (lit.[249]: mp 79-80°C); ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 1.32 (9H, s, ($C\underline{H}_3)_3$); 2.84 and 3.02 (2H, dd, J = 13.0 Hz, J = 4.00Hz, β - H *Phe*); 4.18 (1H, m, α - H *Phe*); 7.09 (1H, d, J 8.4 Hz; N*H*); 7.26 (5H, s, 5H - Ar *Phe*); mass spectrum (C.I.) *m/z* 266 (1%) (M + H), 164 (100%), 192 (47%), 120 (45%), 91 (40%). T.L.C. (A), R_f = 0.62. Anal. Found C, 63.20; H, 7.30; N, 5.25. $C_{14}H_{19}NO_4$ requires C, 63.40; H, 7.15; N, 5.30; specific rotation, $[\alpha]^{23}_{589}$ + 10.4°.

Compound 2. N-(1,1-dimethylethoxycarbonyl)phenylalanine N-allylamide. A 250 mL flask was charged with N (1,1-dimethylethoxycarbonyl)phenylalanine (4.34 g, 16.35 mmol) in dry THF (50 mL) and maintained at 0°C as triethylamine (3.31 g, 32.75 mmol) and 3-methylpropyl chloroformate (2.25 g, 16.35 mmol) were added and the mixture stirred for 1h. A precipitate was filtered off and the filtrate added dropwise to allylamine (9.32 g,

163.50 mmol) in THF (50 mL) and the reaction mixture maintained at 5°C for 16h. The solvent was evaporated and the residue, in CH_2Cl_2, washed with H_2SO_4 (10%), $NaHCO_3$ (10%) and dried before the solvent was evaporated to yield the crude product as a white solid. Recrystallisation from petroleum ether gave N-(1,1-dimethylethoxycarbonyl)phenylalanine N-allylamide (4.35 g, 87%) as white crystals:: mp 96 - 98°C; ^1H NMR ($(CD_3)_2SO$) 270 MHz δ 1.30 (9H, s, $(CH_3)_3O$), 2.70 and 2.90 (2H, dd, J = 10.3 Hz, J = 4.6 Hz, H - β, *Phe*), 3.69 (2H, brs, $HNCH_2CH$), 4.14 (1H, dt, J = 4.0 Hz, H - α, *Phe*), 5.05 (2H, dd, CH_2CHCH_2), 5.74 (1H, m, CH_2CHCH_2), 6.94 (1H, d, J = 8.8 Hz, NH, *Phe*), 7.25 (5H, brs, 5H - Ar, *Phe*), 8.07 (1H, brs, $HNCH_2CH$); mass spectrum (C.I) *m/z* 305 (21%) (M + H), 249 (100%), 205 (47%), 120 (30%). T.L.C. (A), R_f = 0.75. Anal. Found: C, 66.90; H, 7.95; N, 9.10. $C_{17}H_{24}N_2O_3$ requires C, 67.10; H, 7.95; N, 9.20.

Compound 3. Phenylalanine N-allylamide. Trifluoroacetic acid (6.90 g, 60.50 mmol) was added directly to N-(1,1-dimethylethoxycarbonyl)phenylalanine N-allylamide (3.68 g, 12.10 mmol) and the mixture stirred for 4h. CH_2Cl_2 (50 mL) was added followed by water (100 mL) containing K_2CO_3 (16.80 g, 122 mmol). The organic phase was separated and dried and the solvent was evaporated to give phenylalanine N-allylamide (1.70 g, 69%) as a yellow oil: ^1H NMR ($(CD_3)_2SO$) 270 MHz δ 2.89 (2H, dd, J = 13.2 Hz, J = 4.6 Hz, H - β, *Phe*), 3.41 (1H, dd, J = 8.1 Hz, J = 5.2 Hz, H - α, *Phe*), 3.68 (2H, brs, $HNCH_2CH$), 5.02 (2H, m, CH_2CHCH_2), 5.76 (1H, m, CH_2CHCH_2), 7.22 (5H, brs, 5H - Ar, *Phe*), 7.99 (1H, brs, $HNCH_2CH$); mass spectrum (FAB) *m/z* 205 (100%) (M + H), 409 (60%), 120 (15%). T.L.C. (A), R_f = 0.21. Anal. Found C, 70.20; H, 8.05; N, 13.50. $C_{12}H_{16}N_2O$ requires C, 70.55; H, 7.90; N, 13.70.

Compound 4. N-(Allylaminocarbonyl)phenylalanine N-allylamide. Allyl isocyanate (1.30 g, 15.70 mmol) was added to a solution of phenylalanine N-allylamide (1.60 g, 7.85 mmol) in CH_2Cl_2 (40 mL) and stirred for 2h. The solvent was evaporated and the residue was recrystallised from chloroform / light petroleum to afford N-(allylaminocarbonyl)phenylalanine N-allylamide as white crystals (1.84 g, 82%): mp 138 - 139°C; ^1H NMR ($(CD_3)_2SO$) 270 MHz δ 2.78 (2H, dd, J = 13.2 Hz, J = 4.6 Hz, H - β, *Phe*), 3.58 (2H, brs, $CHCH_2NH$, 3), 3.67 (2H, brs, $HNCH_2CH$) 4.38 (1H, q, J = 5.5 Hz, H - α, *Phe*), 5.02 (4H, brs, CH_2CHCH_2), 5.74 (2H, brs, CH_2CHCH_2), 6.16 (2H, brs, NH), 7.19 (5H, brs, 5H - Ar, *Phe*), 8.14 (1H, brs, NH, *Phe*); mass spectrum (C.I) *m/z* 288 (50%) (M + H), 205 (100%),

120 (49%), 231 (40%). T.L.C. (C), $R_f = 0.69$. Anal. Found C, 67.01; H, 7.40; N, 14.75. $C_{16}H_{21}N_3O_2$ requires C, 66.90; H, 7.35; N, 14.60.

Compound 5. N-Allylbenzamide. Benzoyl chloride (1.50 g, 10.65 mmol) was added carefully to allylamine (1.80 g,31.58 mmol) in CH_2Cl_2 (100 mL) and stirred for 1h. The reaction mixture was washed with water, H_2SO_4 (10%), water before evaporation of the dried extract afforded the product N-allylbenzamide (1.38 g, 81%). The product was a pale yellow liquid at 18°C but white crystals were formed at approximately 3°C: ^1H NMR ($CDCl_3$) 400 MHz δ 4.03 (2H, t, J = 5.8 Hz, CH_2), 5.11 (1H, d, J = 10.1 Hz, CH), 5.22 (1H, d, J = 17.1 Hz, CH), 5.89 (1H, ddt, J = 17.1 Hz, J = 10.0 Hz, J = 5.5 Hz, CH), 6.97 (1H, brs, NH), 7.36 (2H, d, J = 7.9 Hz, 2H - Ar, *meta*), 7.45 (1H, t, J = 7.3 Hz, 1H - Ar, *para*), 7.79 (2H, d, J = 8.2 Hz, 2H - Ar, *ortho*); mass spectrum (E.I.) *m/z* 161 (100%) (M), 105 (83%). T.L.C. (A), $R_f = 0.48$. Anal. Found C, 74.70; H, 6.85; N, 8.60. $C_{10}H_{11}NO$ requires C, 74.50; H, 6.90; N, 8.70.

Compound 6. 3-Phenyl-1-allylurea. Allyl isocyanate (1.34 g, 16.15 mmol) and aniline (1.5 g, 16.15 mmol) were dissolved in diethyl ether (50 mL) and stirred for 2h. The diethyl ether was evaporated and the residue, in CH_2Cl_2, was washed twice with aq. H_2SO_4 (10%), and with water. After removing the dried solvent the impure product was triturated with heptane and a brown solid was collected to afford the product 3-phenyl-1-allylurea (0.62 g, 22%): mp 102 - 104°C; ^1H NMR ($CDCl_3$) 400 MHz δ 3.77 (2H, d, J = 5.2 Hz, C*H*$_2$), 5.07 (1H, dd, J = 1.5 Hz, J = 10.2 Hz, C*H*), 5.17 (1H, dd, J = 1.5 Hz, J = 17.1 Hz, C*H*), 5.79 (1H, m, C*H*), 6.99 (1H, t, J = 7.8 Hz, H - Ar, *para*), 7.24 (4H, m, H - Ar, *ortho and meta*), 7.62 (2H, brs, N*H*); mass spectrum (70 eV) *m/z* 176 (10%) (M), 93 (100%), 41 (22%), 56 (18%), (E.I) *m/z* 176 (100%) (M + H), 93 (90%); T.L.C. (C), $R_f = 0.78$. Anal. Found C, 67.60; H, 6.90; N, 16.10. $C_{10}H_{12}N_2O$ requires C, 68.15; H, 6.85; N, 15.90.

Compound 7. N-Phenylpent-4-enamide. Pent-4-enoic acid (3.90 g,39.00 mmol) was treated with $SOCl_2$ (7.00 g, 59.00 mmol) and the mixture boiled under reflux for 5h. The fraction (114 - 118°C) was isolated by distillation to give pent-4-enoyl chloride (1.76 g, 14.90 mmol). This was stirred with an excess of aniline (3.80 g, 40.80 mmol) in CH_2Cl_2 (100mL) at 0°C for 1h. The mixture was washed with H_2SO_4 (10%) (× 5) and water whereupon the CH_2Cl_2 was dried and evaporated to afford the product N-phenylpent-4-enamide as a white solid (2.13 g, 82%): mp 90 - 91°C (lit.[227] mp 90 - 91°C), ^1H NMR

($CDCl_3$) 400 MHz δ 3.77 (2H, d, J = 5.2 Hz, CH_2), 5.07 (1H, dd, J = 1.5 Hz, J = 10.2 Hz, CH), 5.17 (1H, dd, J = 1.5 Hz, J = 17.1 Hz, CH), 5.79 (1H, m, CH), 6.99 (1H, t, J = 7.8 Hz, H - Ar, *para*), 7.24 (4H, m, H - Ar, *ortho and meta*), 7.62 (2H, brs, NH); mass spectrum (E.I.) *m/z* 175 (100%), 93 (90%).

Compound 8. N-(Oxiranylmethyl)benzamide. A mixture of N-(allyl)benzamide (1.07 g, 6.65 mmol) and MCPBA (1.15 g, 6.65 mmol) were boiled under reflux in CH_2Cl_2 (30 mL) for 1h. The reaction was cooled, filtered and the filtrate washed with aq. sodium metabisulphite (10%) twice, $NaHCO_3$ (10%) and water. The CH_2Cl_2 was dried and evaporated to furnish a pale orange oil which was purified by column chromatography (CH_2Cl_2; CH_2Cl_2 : EtOAc; EtOAc) to afford N-(oxiranylmethyl)benzamide (47 mg, 4%) as a pale orange oil. Further analysis revealed: ^1H NMR ($CDCl_3$) 400 MHz δ 3.0 (1H, brs, NH), 3.69 (1H, dd, J = 5.8 Hz, J = 12.2 Hz, OH), 3.8 (1H, dd, J = 12.4 Hz, J = 3.2 Hz, OH), 3.83 (1H, dd, J = 14.5 Hz, J = 7.8 Hz, CH, 1), 4.07 (1H, dd, J = 14.6 Hz, J = 10.0 Hz, CH, 2), 4.80 (1H, m, CH, 3), 7.39 (2H, d, J = 7.6 Hz, 2H - Ar, *meta*), 7.47 (1H, t, J = 7.3 Hz, 1H - Ar, *para*), 7.93 (2H, d, J = 8.2 Hz, 2H - Ar, *ortho*); mass spectrum (E.I) *m/z* 177 (31%) (M), 146 (100%), 77 (70%), 118 (70%), 195 (25%). T.L.C. (C), R_f = 0.31. $C_{10}H_{11}NO_2$.

Compound 9. 4-Acetoxybenzoyl chloride. A mixture of 4-acetoxybenzoic acid (1.5 g, 8.33 mmol) and thionyl chloride (4.5 mL) was stirred at 50°C for 4h. Distillation at 78°C removed any excess thionyl chloride to leave a waxy solid. Further distillation under reduced pressure (140°C, 2 mmHg) afforded the 4-acetoxybenzoyl chloride (1.42 g, 86%) as a white solid: ^1H NMR, ($(CD_3)_2SO$) 270 MHz δ 3.92 (3H, s, CH_3CO), 7.00 (2H, d, J = 8.8 Hz, 2H-Ar 2', 6'), 7.85 (2H, d, J = 8.8 Hz, 2H-Ar 3', 5'), I.R. 1750, 1800 ν cm^{-1}; mass spectrum (C.I.) *m/z* 153 (100%) (M + H), 135 (20%). $C_9H_7O_3Cl$.

Compound 10. N-(4-(Methoxybenzoyl)glycine methyl ester. Glycine methyl ester hydrochloride (2.65 g, 21.10 mmol) was dissolved in CH_2Cl_2 (100 mL) containing triethylamine (3.56 g, 35.16 mmol). Dropwise 4-methoxybenzoyl chloride (3.00 g, 17.58 mmol) was added and the mixture stirred for 16h. Using aq. H_2SO_4 (5%), Na_2CO_3 (10%) and water the CH_2Cl_2 was washed, dried and evaporated to yield the product N-(4-(methoxybenzoyl)glycine methyl ester (3.39 g, 86%) as a white solid: mp 110 - 113°C (lit. [338] mp 110 - 115°C); ^1H NMR ($(CD_3)_2SO$) 270 MHz δ 3.65 (3H, s, OCH_3), 3.81 (3H, s,

$C\underline{H}_3OAr$), 4.02 (2H, d, J = 5.9 Hz, $C\underline{H}_2$), 7.03 (2H, d, J = 8.9 Hz, 2H-Ar 2', 6'), 7.88 (2H, d, J = 8.9 Hz, 2H-Ar 3', 5'), 8.84 (1H, t, J = 6.0 Hz, N$\underline{H}$); mass spectrum (E.I.) *m/z* 223 (100%) (M + H), 135 (51%), 164 (11%), 191 (10%). T.L.C. (A), $R_f = 0.55$. Anal. Found C, 59.10; H, 5.90; N, 6.30. $C_{11}H_{13}NO_4$ requires C, 59.20; H, 5.85; N, 6.25.

Compound 11. N-(2-(4-Hydroxyphenyl)ethyl)-4-methoxybenzamide. Carefully, 4-methoxybenzoyl chloride (1.24 g, 7.30 mmol) was added to a solution of triethylamine (0.74 g, 7.30 mmol) and 2-(4-hydroxyphenyl)ethylamine (1.00 g, 7.30 mmol) in DMF (12 mL) and the solution was stirred for 1h. Water was added and the precipitate was collected and dried under vacuum to afford N-(2-(4-hydroxyphenylethyl)-4-methoxybenzamide (1.51 g, 76%) as a white solid: mp 166 - 168°C; ^{1}H 270 NMR ($(CD_3)_2SO$) δ 2.73 (2H, t, J = 7.4 Hz, $CH_2C\underline{H}_2$), 3.41 (2H, m, $CH_2C\underline{H}_2$), 3.78 (3H, s, $(OC\underline{H}_3)$, 6.70 (2H, d, J = 8.3 Hz, 2H-Ar' 3', 5'), 6.97 (2H, d, J = 8.8 Hz, 2H-Ar 3', 5'), 7.03 (2H, d, J = 8.4 Hz, 2H-Ar' 2', 6'), 7.83 (2H, d, J = 8.6 Hz, 2H-Ar 2', 6'), 8.42 (1H, m, NH), 9.25 (1H, s, OH); mass spectrum (C.I.) *m/z* 271 (8%) (M + H), 135 (100%), 152 (65%), 120 (52%). T.L.C. (A), $R_f = 0.40$. Anal. Found C, 71.20; H, 6.70; N, 4.85. $C_{16}H_{17}NO_3$ requires C, 70.80; H, 6.30; N, 5.15.

Compound 12. 4-Methoxy-N-(2-(4-methoxyphenyl)ethyl)benzamide. With care, 4-methoxybenzoyl chloride (3.50 g, 20.50 mmol) was added to 2-(4-methoxyphenyl)ethylamine (3.10 g, 20.50 mmol), triethylamine (2.07 g, 20.50 mmol) and DMAP (5 mg) in CH_2Cl_2 (50 mL) and stirred for 5h. The crude product, in CH_2Cl_2 (100 mL), was washed with water, H_2SO_4 (10%) (twice), $NaCO_3$ (10%) and water after which the solvent was dried and evaporated to afford a solid. Recrystallisation from ethanol / water furnished 4-methoxy-N-(2-(4-methoxyphenyl)ethyl)benzamide (5.35 g, 91%) as white crystals: mp 160 - 162°C; ^{1}H 270 NMR ($CDCl_3$) δ 2.85 (2H, t, J = 7.0 Hz. $CH_2C\underline{H}_2$), 3.65 (2H, q, J = 7.0 Hz, $C\underline{H}_2CH_2$), 3.79 (3H, s, $ArOC\underline{H}_3$), 3.82 (3H, s, $ArOC\underline{H}_3$), 6.15 (1H, m, NH), 6.85 (2H, d, J = 8.4 Hz, 2H - Ar' 2', 6'), 6.90 (2H, d, J = 8.4 Hz, 2H - Ar 2', 6'), 7.15 (2H, d, J = 8.6 Hz, 2H - Ar' 3', 5'), 7.65 (2H, d, J = 8.8 Hz, 2H - Ar 3', 5'); mass spectrum (C.I.) *m/z* 286 (100%) (M + H), 134 (53%), 152 (16%), 69 (19%). T.L.C. ($CHCl_3$: MeOH, 15 : 1), R_f = 0.70. Anal. Found C, 71.20; H, 6.70; N, 4.85. $C_{17}H_{19}NO_3$ requires C, 70.85; H, 6.30; N, 5.15.

Compound 13. 4-Hydroxy-N-(2-(4-hydroxyphenyl)ethyl)benzamide. 4-Methoxy-N-(2-(4-methoxyphenyl)ethylbenzamide (2.00 g, 7.01 mmol) was boiled under reflux for 48h with

HBr / acetic acid (30%) (20 mL, 74.15 mmol). After the solvent had been evaporated to leave a volume of 10 mL, water was added. The crude product formed as a precipitate which was collected. The solid was further purified by column chromatography (CH_2Cl_2 : EtOAc; EtOAc) and the organic solvent was evaporated from an eluted fraction to give 4-hydroxy-N-(2-(4-hydroxyphenyl)ethyl)benzamide as a brown solid (0.34 g, 17%): mp 210 - 212°C; ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 2.70 (2H, t, J = 7.5 Hz, $CH_2C\underline{H_2}$), 3.35 (2H, q, J = 7.5 Hz, $C\underline{H_2}CH_2$), 6.70 (2H, d, J = 8.5 Hz, 2H - Ar' 2' 6'), 6.80 (2H, d, J = 8.6 Hz, 2H - Ar 2', 6'), 7.00 (2H, d, J = 8.5 Hz, 2H - Ar' 3', 5'), 7.70 (2H, d, J = 8.8 Hz, 2H - Ar 3', 5'), 8.25 (1H, t, J = 5.4 Hz, NH), 9.00 (1H, brs, OH), 10.00 (1H, brs, OH); mass spectrum (C.I.) *m/z* 258 (100%) (M + H), 69 (38%), 120 (35%), 138 (34%). T.L.C. (A), $R_f = 0.30$. Anal. Found C, 70.35; H, 6.20; N, 5.15. $C_{15}H_{15}NO_3$ requires C, 70.00; H, 5.90; N, 5.45.

Compound 14. (Identical to *Compound 13*). **4-Hydroxy-N-(2-(4-hydroxyphenyl)ethyl)-benzamide.** 4-Methoxy-N-(2-(4-methoxyphenyl)ethyl)benzamide (2.00 g, 7.00 mmol) was boiled under reflux for 30 min with BBr_3 / CH_2Cl_2 (25 mL, 1.0M). The CH_2Cl_2 was evaporated and the residue washed with water before it was recrystallised from isopropanol / pet. ether to give the product 4-hydroxy-N-(2-(4-hydroxyphenyl)ethyl)benzamide (1.30 g, 72%) as a white solid with properties as above.

Compound 15. N-(N-(N-(N-(4-Methoxybenzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide. N-(4-Methoxybenzoyl)glycine pentafluorophenyl ester (5.22 g, 13.95 mmol), HOBt (200 mg) and diisopropylethylamine (2.13 g, 16.45 mmol) were dissolved in CH_2Cl_2 (150 mL). N-(N-(Phenylalanyl)leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide (5.95 g,12.65 mmol) in CH_2Cl_2 (50 mL) was added dropwise to the solution and stirred at 35°C for 24h with DMAP (30 mg). The CH_2Cl_2 was evaporated to give the crude product which was purified using chromatography (3:1 (EtOAc: CH_2Cl_2); 7:1 ($CHCl_3$: MeOH)). The solvent was evaporated and the product N-(N-(N-(N-(4-methoxybenzoyl)glycyl)phenylalanyl)leucyl)gycine N-(2-(4-methoxyphenyl)ethyl)amide obtained as white crystals: mp 82 - 84°C; ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 0.86 (3H, d, J = 6.1 Hz, CH_3, *Leu*), 0.90 (3H, d, J = 6.4 Hz, CH_3, *Leu*), 1.54 (2H, brs, β - H, *Leu*), 1.56 (1H, m, γ - H, *Leu*), 2.65 (2H, t, J = 7.5 Hz, $HNCH_2C\underline{H_2}$,), 2.85 (1H, dd, J = 9.5 Hz, β - H, *Phe*), 3.07 (1H, dd, J = 9.7 Hz, β - H, *Phe*), 3.25 (2H, q, J = 7.0 Hz, $HNC\underline{H_2}CH_2$), 3.63 (2H, d, J = 5.8 Hz, CH_2 *Gly'*), 3.72 (3H, s, OCH_3), 3.77 (1H, d, J = 5.8 Hz, CH, *Gly*), 3.83 (3H, s, CH_3), 3.87 (1H, d, J = 5.8 Hz, CH_2, *Gly*), 4.27 (1H, m, α - H, *Leu*), 4.57 (1H, m, α - H,

Phe), 6.84 (2H, d, J = 8.9 Hz, 2H - Ar' 2', 6'), 7.01 (2H, d, J = 8.9 Hz, 2H - Ar 2', 6'), 7.12 (2H, d, J = 8.9 Hz, 2H - Ar' 3', 5'), 7.24 (5H, brs, 5H - Ar, *Phe*), 7.81 (1H, brt, $\underline{H}NCH_2CH_2$), 7.85 (2H, d, J = 8.9 Hz, 2H - Ar 3', 5'), 8.04 (1H, brt, NH *Gly*), 8.14 (1H, d, J = 8.6 Hz, NH, *Leu*), 8.18 (1H, d, J = 7.7 Hz, NH, *Phe*), 8.66 (1H, brt, NH - *Gly*); mass spectrum (FAB [+]) *m/z* 660 (10%) (M + H), 135 (100%), 120 (40%), 86 (35%). (FAB [-]) *m/z* 658 (100%) (M - H), 207 (9%), 154 (4%), 450 (5%). T.L.C. (A), R_f = 0.20. $C_{36}H_{45}N_5O_7$.

Compound 16. Phenylmethyl 4-(phenylmethoxy)benzoate. A mixture of 4-hydroxybenzoic acid (27.60 g, 200 mmol), chloromethylbenzene (57.0 g, 450 mmol), K_2CO_3 (50 g) and NaI (25 g) was boiled under reflux in MeCN (500 mL) for 16h. The suspension was filtered and the solvent was evaporated to give a solid which was recrystallised from ethanol to give phenylmethyl 4-(phenylmethoxy)benzoate (48.8 g, 76%) as white crystals: mp 114 - 116°C, (lit. [339] mp 115.5 - 116.5°C); ^{1}H NMR ($CDCl_3$) 270 MHz δ 5.12 (2H, s, BnC$\underline{H}_2$O), 5.34 (2H, s, OC$\underline{H}_2$Bn), 7.00 (2H, d, J = 8.9 Hz, 2H - Ar 2', 6'), 7.35 - 7.46 (10H, m, $\underline{Bn}CH_2O$, twice), 8.03 (2H, d, J = 8.8 Hz, 2H - Ar 3', 5'); mass spectrum (C.I.) *m/z* 318 (M), 227, 211, 107, 91. Anal. Found C, 79.45; H, 5.65. $C_{21}H_{18}O_3$ requires C, 79.20; H, 5.70.

Compound 17. 4-(Phenylmethoxy)benzoic acid. Phenylmethyl 4-(phenylmethoxy)-benzoate (48.8 g, 150 mmol) was boiled under reflux with aq. NaOH (2M; 250 mL) and ethanol (250 mL) for 4h. The ethanol was evaporated and water (1000 mL) was added. The white solid was collected by filtration and stirred with aq. H_2SO_4 (2M; 300 mL) for 1h at 65°C and then extracted with warm EtOAc. The solution was dried and the solvent evaporated to give 4-(phenylmethoxy)benzoic acid (27.15 g, 80%). The filtrate was washed twice with diethyl ether, acidified with H_2SO_4 (2M) and extracted with diethyl ether. Evaporation of the diethyl ether gave the product 4-(phenylmethoxy)benzoic acid (6.0 g, 18%). The total yield was 98% of white crystals: mp 186 - 187°C, (lit. [233] mp 186 - 187° C); ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 5.18 (2H, s, BnC$\underline{H}_2$O), 7.09 (2H, d, J = 8.8 Hz, 2H - Ar 2', 6'), 7.30 - 7.46 (5H, m, $\underline{Bn}CH_2O$), 7.89 (2H, d, J = 8.8 Hz, 2H - Ar 3', 5'), 12.60 (1H, brs, OH); mass spectrum (C.I.) *m/z* 228 (M), 183, 159, 121, 91; IR ν cm^{-1} 1690, 1600 and 1175; $C_{14}H_{12}O_3$.

Compound 18. 4-(Phenylmethoxy)benzoyl chloride. To a stirred solution of 4-(phenylmethoxy)benzoic acid (500 mg, 2.2 mmol) and oxalyl chloride (280 mg, 2.2 mmol) in

1,4-dioxan (25 mL), DMF (25 mg) was added. After the evolution of a gas had ceased, the solvent and catalyst were evaporated and the residue was recystallised from light petroleum to give 4-(phenylmethoxy)benzoyl chloride (460 mg, 85%) as a white solid: mp 104 - 106°C (lit. [233] mp 104 - 106°C); IR ν cm^{-1} 1775, 1600 and 1175 ; ^1H NMR ($(CD_3)_2SO$) 270 MHz δ 5.15 (2H, s, BnCH_2O), 7.03 (2H, d, J = 8.9 Hz, 2H - Ar 2', 6'), 7.37 - 7.42 (5H, m, *Bn*CH_2O), 8.07 (2H, d, J = 8.8 Hz, 2H - Ar 3', 5'); $C_{14}H_{11}O_2Cl$.

Compound 19. 1-(2-Nitroethenyl)-4-(phenylmethoxy)benzene. Nitromethane (16.1 g, 264 mmol) was added to 4-(phenylmethoxy)benzaldehyde (28 g, 132 mmol) in EtOH (900 mL) at 5°C. NaOH (13.2 g, 330 mmol) in EtOH (200 mL) was added dropwise and the mixture was stirred for 30 min at 5°C. The reaction mixture was poured into a mixture of aq. HCl (9M; 136 mL) and water (208 mL). The precipitate was collected and recrystallised from EtOH to give 1-(2-nitroethenyl)-4-(phenylmethoxy)benzene (14.0 g, 42%) as a yellow solid: mp 120 - 121°C (lit. [341] mp 120 - 121°C); ^1H NMR ($CDCl_3$) δ 5.02 (2H, s, ArCH_2O), 6.92 (2H, d, J = 8.8 Hz, 2H - Ar 3', 5'), 7.29 (5H, m, 5H - *Ar* OCH_2*Ph*), 7.38 (2H, d, J = 8.8 Hz, 2H - Ar 2', 6'), 7.43 (1H, d, J = 13.5 Hz, C*H*$CHNO_2$), 7.85 (1H, d, J = 13.5 Hz, CHC*H*NO_2); T.L.C. (heptane : diethyl ether, 1 : 1), R_f = 0.58. $C_{15}H_{13}NO_3$.

Compound 20. 2-(4-(Phenylmethoxy)phenyl)ethylamine. Lithium aluminium hydride (8.48 g, 223 mmol) was suspended in dry diethyl ether (600 mL). Using a Soxhlet apparatus, 1-(2-nitroethenyl)-4-(phenylmethoxy)benzene (13.9 g, 55 mmol) was extracted into the mixture and boiled under reflux for 16 h. Water (7.38 mL) was added, followed by aq. NaOH (20%; 5.53 mL) and water (27.8 mL). The suspension was filtered and the solvent was evaporated to give 2-(4-(phenylmethoxy)phenyl)ethylamine (11.25 g, 91%) as a white solid: mp 44 - 46°C (lit. [341] mp 196 - 206°C as the chloride salt); ^1H 400 NMR ($(CD_3)_2SO$) δ 2.56 (2H, t, J = 7.1 Hz, C*H*$_2CH_2$), 2.71 (2H, t, J = 7.3 Hz, CH_2C*H*$_2$), 5.05 (2H, s, CH_2, *Bn*), 6.91 (2H, d, J = 8.8 Hz, 2H - Ar 2' - 6'), 7.10 (2H, d, J = 8.8 Hz, 2H - Ar 3' - 5'), 7.31 - 7.44 (5H, m, Ar, *Bn*); mass spectrum (70 E.I.) *m/z* 227 (4%) (M), 91 (100%), 198 (40%), 30 (31%); (C.I.) m/z 228 (M^+) (86%), 91 (100%), 198 (61%), 212 (56%)T.L.C. (A), R_f = 0.23. $C_{15}H_{17}NO$.

Compound 21. N-(N-(4-(Phenylmethoxy)benzoyl)glycyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide. Dry CH_2Cl_2 (14 mL) containing N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride (140 mg, 0.437 mmol) and N,N-

diisopropylethylamine (118.61 mg, 0.918 mmol) was stirred for 6h as N-(4-(phenylmethoxy)benzoyl)glycine pentafluorophenyl ester was added dropwise to the mixture (197.3 mg, 0.437 mmol). A precipitate was removed by filtration before the CH_2Cl_2 was evaporated and the residue applied to a chromatography column, (EtOAc : $CHCl_3$ [1 : 1]). A single product was eluted and the solvent evaporated to give N-(N-(4-(phenylmethoxy)-benzoyl)glycyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (19 mg, 8%): ^1H NMR ($(CD_3)_2SO$) 270 MHz δ 2.65 (2H, t, J = 7.5 Hz, $HNCH_2C\underline{H}_2$,), 3.23 (2H, q, J = 6.8 Hz, $HNC\underline{H}_2CH_2$), 3.67 (2H, d, J = 5.5 Hz, CH_2 *Gly'*), 3.87 (2H, d, J = 5.5 Hz, CH_2 *Gly*), 5.03 (2H, brs, CH_2, $OC\underline{H}_2Bn$), 5.15 (2H, brs, CH_2, $OC\underline{H}_2Bn$), 6.83 (2H, d, J = 8.3 Hz, 2H - Ar' 2', 6'), 7.01 (2H, d, J = 8.8 Hz, 2H - Ar 2', 6'), 7.12 (2H, d, J = 8.5 Hz, 2H - Ar' 3', 5'), 7.43 (10H, m, $OCH_2\underline{Bn}$), 7.85 (1H, m, $N\underline{H}CH_2CH_2$), 7.88 (3H, d, J = 8.9, 2H - Ar 3' - 5, 1H - $\underline{H}NCH_2CH_2$), 8.18 (1H, brt, NH *Gly'*), 8.73 (1H, brt, NH - *Gly*); mass spectrum (F.A.B. [+]) *m/z* 552 (15%), 285 (100%), 391 (23%); (F.A.B. [-]) *m/z* 550 (40%), 97 (100%), 457 (29%); T.L.C. (C), $R_f = 0.74$; $C_{33}H_{33}N_3O_5$.

Compound 22. N-(N-(N-(4-(Phenylmethoxy)benzoyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide. N,N-Diisopropylethylamine (200 mg, 1.575 mmol) was added to N-(N-(N-phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)-ethyl)amide hydrochloride (403 mg, 0.693 mmol) and 4-(phenylmethoxy)benzoyl chloride suspended in dry CH_2Cl_2 (50 mL) and was stirred for 3h. The CH_2Cl_2 was evaporated and the residue was further purified by column chromatography (Et_2O; EtOAc) to afford N-(N-(N-(4-(phenylmethoxy)benzoyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (450 mg, 94%) as a white solid: mp 157 - 159°C; ^1H NMR ($(CD_3)_2SO$) 400 MHz δ 0.76 (3H, d, J = 6.3 Hz, CH_3 *Leu*, Isomer 1), 0.82 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 1), 0.83 (3H, d, J = 6.3 Hz, CH_3 *Leu*, Isomer 2), 0.88 (3H, d, J = 6.8 Hz, CH_3 *Leu*, Isomer 2), 1.33 (1H, m, γ - H *Leu*, Isomer 1), 1.45 (2H, t, J = 7.3 Hz, β - H *Leu*, Isomer 1), 1.56 (2H, t, J = 7.3 Hz, β - H *Leu*, Isomer 2), 1.61 (1H, m, γ - H *Leu*, Isomer 2), 2.54 (2H, t, J = 7.6 Hz, $HNCH_2C\underline{H}_2$, Isomer 1), 2.62 (2H, t, J = 7.6 Hz, $HNCH_2C\underline{H}_2$, Isomer 2), 3.05 (2H, dd, J = 7.8 Hz, β - H *Phe*, Isomer 1), 3.10 (2H, dd, J = 7.8, J = buried, β - H *Phe*, Isomer 2), 3.12 (2H, q, $HNC\underline{H}_2CH_2$, Isomer 1), 3.22 (2H, q, J = 6.8 Hz, $HNC\underline{H}_2CH_2$, Isomer 2), 3.66 (2H, m, CH_2 *Gly*, Isomer 1), 3.70 (2H, d, J = 6.3 Hz, CH_2 *Gly*, Isomer 2), 4.17 (1H, q, J = 7.5 Hz, α - H *Leu*, Isomer 1), 4.28 (1H, q, J = 7.5 Hz, α - H *Leu*, Isomer 2), 4.67 (1H, q, J = 6.7 Hz, α - H *Phe*, Isomer 1), 4.69 (1H, m, α - H *Phe*, Isomer 2), 5.03 (4H, brs, CH_2, $OC\underline{H}_2Ph'$, Isomer 1 and 2), 5.12 (4H, brs, CH_2, $OC\underline{H}_2Ph$, Isomer 1 and 2), 6.87 (4H, d, J = 8.6 Hz, 2H - Ar' 2', 6'), 7.02 (4H, d, J = 8.6 Hz,

2H - Ar 2', 6'), 7.22 - 7.45 (30H, m, 5H - Ar *Phe*, Isomer 1 and 2, 10H - Ar_OCH$_2$*Bn*, Isomer 1 and 2), 7.30 (4H, approx' d, 2H - Ar' 3', 5'), 7.59 (1H, t, J = 5.6 Hz, *H*NCH$_2$CH$_2$, Isomer 1), 7.80 (4H, d, J = 6.7 Hz, 2H - Ar 3', 5'), 7.81 (1H, buried, *H*NCH$_2$CH$_2$, Isomer 2), 8.12 (1H, t, J = 5.8 Hz, NH *Gly*), 8.19 (1H, d, J = 7.8 Hz, NH *Leu*, Isomer 2), 8.26 (1H, t, J = 5.9 Hz, NH *Gly*), 8.42 (1H, d, J = 7.8 Hz, NH *Phe*, Isomer 2), 8.43 (1H, d, J = 7.3 Hz, NH *Leu*, Isomer 1), 8.57 (1H, d, J = 6.8 Hz, NH *Phe*, Isomer 1); mass spectrum (FAB [+]) *m/z* 755 (8%) (M + H), 777 (2%). (FAB [-]) *m/z* 753 (100%) (M - H), 663 (20%), 571 (2%). Accurate mass. Found 756.3892, calculated $C_{46}H_{52}N_4O_6$ requires 756.3887. T.L.C. ($CHCl_3$: MeOH, 9 : 1), R_f = 0.50.

Compound 23. N-(N-(N-(N-(4-(Phenylmethoxy)benzoyl)glycyl)phenylalanyl)leucyl)-glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide. N-(N-(N-Phenylalanyl leucyl)-glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride (165 mg, 0.284 mmol) was treated with N,N-diisopropylethylamine (100 mg, 0.774 mmol), DMAP (10 mg) and HOBt (10mg) in dry CH_2Cl_2 (15 mL) until dissolved. N-(4-(Phenylmethoxy)benzoyl)glycine pentafluorophenyl ester (117 mg, 0.258 mmol) in CH_2Cl_2 (10mL) was added dropwise and the mixture stirred for 5h. The solvent was evaporated and the residue was purified by column chromatography ($CHCl_3$: MeOH (50:1)) to afford N-(N-(N-(N-(4-(phenyl-methoxy)benzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)-ethyl)amide (170 mg, 81%) as a glass: softening at 68 - 70°C; ^{1}H 400 MHz NMR ($(CD_3)_2SO$) δ 0.75 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 1), 0.79 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 1), 0.83 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 2), 0.87 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 2), 1.45 (2H, t, J = 7.2 Hz, β - H *Leu*, Isomer 1), 1.51 (2H, t, J = 7.3 Hz, β - H *Leu*, Isomer 2), 1.62 (1H, m, γ - H *Leu*, Isomer 1), 1.73 (1H, m, γ - H *Leu*, Isomer 2), 2.62 (2H, t, J = 7.0 Hz, HNCH$_2$C*H*$_2$, Isomer 1), 2.64 (2H, t, J = 7.0 Hz, HNCH$_2$C*H*$_2$, Isomer 2), 2.78 - 2.88 (2H, m, β - H *Phe*, Isomer 1), 2.97 - 3.07 (2H, m, β - H *Phe*, Isomer 2), 3.22 (4H, q, J = 7.0 Hz, HNC*H*$_2$CH$_2$, Isomer 1 and 2), 3.62 (2H, d, J = 6.7 Hz, CH_2 *Gly*, Isomer 1), 3.66 (2H, d, J = 6.7 Hz, CH_2 *Gly'*, Isomer 1), 3.81 (2H, m, CH_2 *Gly*, Isomer 2), 3.85 (2H, d, J = 6.7 Hz, CH_2 *Gly'*, Isomer 2), 4.22 (2H, m, α - H *Leu*, Isomer 1 and 2), 4.54 (2H, m, α - H *Phe*, Isomer 1 and 2), 5.04 (4H, brs, CH_2, OC*H*$_2$Ph', Isomer 1 and 2), 5.16 (4H, brs, CH_2, OC*H*$_2$Ph, Isomer 1 and 2), 6.90 (4H, d, J = 8.6 Hz, 2H - Ar' 2', 6'), 7.06 (4H, d, J = 8.6 Hz, 2H - Ar 2', 6'), 7.20 (4H, d, J = 8.7 Hz, 2H - Ar' 3', 5'),7.29 - 7.46 (30H, m, 10H - Ar *Phe*, Isomer 1 and 2, 10H - Ar_OCH$_2$*Bn*, Isomer 1 and 2, 10H - Ar OCH$_2$*Bn'*, Isomer 1 and 2), 7.77 (1H, brs, *H*NCH$_2$CH$_2$, Isomer 1), 7.83 (4H, d, J = 8.8 Hz, 2H - Ar 3', 5'); 8.00 - 8.60 (1H, *H*NCH$_2$CH$_2$, Isomer 2), (1H, t, J = 5.8 Hz, NH *Gly'*) (1H, t, J = 5.8 Hz, NH

Gly), (1H, t, J = 5.8 Hz, NH *Gly'*), (1H, d, J = 7.8 Hz, NH *Leu*, Isomer 2), (1H, t, J = 5.9 Hz, NH *Gly*), (1H, d, J = 7.8 Hz, NH *Phe*, Isomer 2), (1H, d, J = 7.3 Hz, NH *Leu*, Isomer 1), (1H, d, J = 6.8 Hz, NH *Phe*, Isomer 1); T.L.C. (EtOAc : MeOH [9 : 1), R_f = 0.4. $C_{48}H_{53}N_5O_7$.

Compound 24. N-(N-(N-(4-Hydroxybenzoyl)phenylalanyl)leucyl)glycine N-(2-(4-hydroxyphenyl)ethyl)amide. N-(N-(N-(4-(Phenylmethoxy)benzoyl)phenylalanyl)-leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (122 mg, 0.161 mmol) was dissolved in absolute alcohol (10 mL) and stirred vigorously with palladium/charcoal (25 mg, (Pd / C, 10%)) under hydrogen for 16h. After filtering the suspension through Celite®, the EtOH was evaporated to furnish the product N-(N-(N-(4-(hydroxy)benzoyl)phenyl-alanyl)leucyl)glycine N-(2-(4-hydroxyphenyl)ethyl)amide (85 mg, 91%) as a white solid: mp 104 - 106°C; ^{1}H NMR ($(CD_3)_2SO$) 400 MHz δ 0.80 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 1), 0.86 (3H, d, J = 6.7 Hz, CH_3 *Leu*, Isomer 1), 0.88 (3H, d, J = 7.3 Hz, CH_3 *Leu*, Isomer 2), 0.93 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 2), 1.36 (1H, m, γ - H *Leu*, Isomer 1), 1.48 (2H, t, J = 7.3 Hz, β - H *Leu*, Isomer 1), 1.56 (2H, t, J = 7.0 Hz, β - H *Leu*, Isomer 2), 1.65 (1H, m, γ - H *Leu*, Isomer 2), 2.51 (2H, $HNCH_2C\underline{H}_2$, Isomer 1), 2.62 (2H, t, J = 7.6 Hz, $HNCH_2C\underline{H}_2$, Isomer 2), 3.08 (4H, m, β - H *Phe*, Isomer 1 and 2), 3.14 (2H, m, $HNC\underline{H}_2CH_2$, Isomer 1), 3.25 (2H, m, $HNC\underline{H}_2CH_2$, Isomer 2), 3.66 (2H, m, CH_2 *Gly*, Isomer 1), 3.70 (2H, d, J = 5.8 Hz, CH_2 *Gly*, Isomer 2), 4.19 (1H, q, J = 7.3 Hz, α - H *Leu*, Isomer 1), 4.31 (1H, q, J = 7.3 Hz, α - H *Leu*, Isomer 2), 4.69 (1H, q, J = 7.6 Hz, α - H *Phe*, Isomer 1), 4.73 (1H, m, α - H *Phe*, Isomer 2), 6.70 (4H, approx' t, 2H - Ar' 2', 6'), 6.81 (4H, approx' d, 2H - Ar 2', 6'), 7.00 (4H, approx' d, 2H - Ar' 3', 5'), 7.30 - 7.38 (10H, brs, 5H - Ar *Phe*, Isomer 1 and 2), 7.60 (1H, brs, $\underline{H}NCH_2CH_2$, Isomer 1), 7.70 (4H, approx' dd, 2H - Ar 3', 5'), 7.85 (1H, brt, $\underline{H}NCH_2CH_2$, Isomer 2), 8.17 (1H, brt, NH *Gly*), 8.21 (1H, d, J = 7.6 Hz, NH *Leu*, Isomer 2), 8.31 (1H, brt, NH *Gly*, Isomer ?), 8.34 (1H, d, J = 8.2 Hz, NH *Phe*, Isomer 2), 8.46 (1H, d, J = 6.4 Hz, NH *Leu*, Isomer 1), 8.50 (1H, d, J = 6.7 Hz, NH *Phe*, Isomer 1), 9.22 (1H, s, OH), 10.06 (1H, s, OH); mass spectrum (FAB [+]) *m/z* 575 (20%) (M + H), 597 (10%), 121 (100%). (FAB [-]) *m/z* 573 (100%) (M - H), 453 (18%). T.L.C. (C), R_f = 0.63. $C_{32}H_{38}N_4O_6$.

Compound 25. N-(N-(N-(N-(4-Hydroxybenzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-hydroxyphenyl)ethyl)amide. N-(N-(N-(N-(4-(Phenylmethoxy)benzoyl)glycyl)-phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (444 mg, 0.547

mmol) dissolved in EtOH (45 mL) was stirred vigorously over palladium/ charcoal catalyst (50 mg, (Pd/ C, 10%)) and hydrogen for 12 h. The suspension was filtered through Celite® and the solvent was evaporated from the filtrate to afford the product N-(N-(N-(N-(4-hydroxybenzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-hydroxyphenyl)ethyl)amide (304 mg, 88%) as an oil; mass spectrum (FAB [-]) *m/z* 630 (40%) (M - H), 188 (100%), 489 (25%). T.L.C. (C), $R_f = 0.46$. $C_{34}H_{41}N_5O_7$.

Compound 26. N-(4-(Methoxybenzoyl)glycine. N-(4-(Methoxybenzoyl)glycine methyl ester (2.00 g, 8.92 mmol) was boiled under reflux for 2h in MeOH (50 mL) containing NaOH (2.00 g, 50 mmol). Once the methanol had been evaporated the residue was dissolved in water, washed with CH_2Cl_2 and acidified (H_2SO_4 (10%)). The precipitate was extracted into CH_2Cl_2, the solution was dried and the solvent was evaporated to afford N-(4-(methoxybenzoyl)glycine (1.85 g, 98%). A small sample was recrystallised from water: mp 173-175°C softening at 168°C (lit. [342] mp 168°C); ^{1}H NMR, ($(CD_3)_2SO$) 270 MHz δ 3.79 (3H, s, CH_3O), 3.92 (2H, d, J = 5.8 Hz, CH_2), 7.00 (2H, d, J = 8.8 Hz, 2H-Ar 2', 6'), 7.85 (2H, d, J = 8.8 Hz, 2H-Ar 3', 5'), 8.71 (1H, t, J = 5.9 Hz, HN); mass spectrum (C.I.) *m/z* 153 (100%), 135 (20%). T.L.C. (A), $R_f = 0.20$. Anal. Found C, 52.10; H, 5.65; N, 5.90. $C_{10}H_{11}NO_4$ requires C, 52.85; H, 5.75; N, 6.15.

Compound 27. N-(4-Methoxybenzoyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide. A mixture of N-(4-methoxybenzyl)glycine (0.5 g, 2.40 mmol) and DCC (0.45 g, 2.20 mmol) was stirred at 0°C in THF (20 mL) for 30 mins. 2-(4-(methoxyphenyl)ethylamine (0.30 g, 2.00 mmol) was added to the suspension which was stirred for a further 16h at 5°C. A precipitate was removed and the organic solvent evaporated under reduced pressure. A residue was redissolved in EtOAc and washed with H_2SO_4 (10%), $NaHCO_3$ and water before the dried solvent was removed to give N-(4-methoxybenzoyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide (0.34 g, 50%) as a white solid: mp 172 - 174°C; ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 2.65 (2H, t, J = 7.5 Hz, $HNCH_2C\underline{H}_2$,), 3.23 (2H, q, J = 6.8 Hz, $HNC\underline{H}_2CH_2$), 3.70 (3H, s, OCH_3'), 3.81 (3H, s, OCH_3), 3.87 (2H, d, J = 5.5 Hz, CH_2 *Gly*), 6.83 (2H, d, J = 8.3 Hz, 2H - Ar' 2', 6'), 7.01 (2H, d, J = 8.8 Hz, 2H - Ar 2', 6'), 7.12 (2H, d, J = 8.5 Hz, 2H - Ar' 3', 5'), 7.85 (1H, m, $N\underline{H}CH_2CH_2$), 7.88 (3H, d, J = 8.9, 2H - Ar 3' - 5), 8.73 (1H, brt, NH *Gly*); mass spectrum (C.I.) *m/z* 343 (85%) (M + H), 134 (100%), 152 (60%), 192 (30%). T.L.C. (A), $R_f = 0.23$. $C_{19}H_{22}N_2O_4$.

Compound 28. N-(4-Methoxybenzoyl)glycine pentafluorophenyl ester. Dry THF (50 mL) containing N-(4-methoxybenzoyl)glycine (1.00 g, 4.75 mmol) and DCC (0.98 g, 4.75 mmol) was stirred at 0°C for 30 min. Pentafluorophenol (0.87 g, 4.75 mmol) in dry THF (20 mL) was added dropwise and the mixture was stirred for 2h at 0°C. After maintaining the mixture at 5°C for 16h the suspension was filtered and the solvent was evaporated to give a residue which was dissolved in CH_2Cl_2 and washed with sat. $NaHCO_3$. The CH_2Cl_2 was dried and evaporated to give N-(4-methoxybenzoyl)glycine pentafluorophenyl ester (1.66 g, 93%) obtained as a yellow oil which soon crystallised to give a pale yellow solid: mp 130 - 132°C; ^1H, NMR (CD_3COCD_3) 270 MHz δ 3.86 (3H, brs, OMe), 4.57 (2H, d, J = 5.9 Hz, CH_2), 7.02 (2H, d, J = 6.8 Hz, 2H-Ar 2', 6'), 7.93 (2H, d, J = 6.6 Hz, 2H-Ar 3', 5'), 8.36 (1H, brs, NH); ^{19}F NMR 400 MHz ($(CD_3)_2SO$) δ -162.66 (2F, dd, J = 23.0 Hz, J = 19.5 Hz, F - *meta*), -157.98 (1F, tt, J = 23.0 Hz, F - *para*), -153.27 (2F, dd, J = 19.5 Hz, F - *ortho*); mass spectrum (C.I.) *m/z* 376 (17%) (M + H), 192 (100%), 135 (80%), 69 (52%). T.L.C. (A), R_f = 0.65. Anal. Found C, 51.10; H, 2.60; N, 3.70. $C_{16}H_{10}NO_4F_5$ requires C, 51.20; H, 2.70; N, 3.75.

Compound 29. N-(N-(4-(Methoxybenzoyl)glycyl)glycine methyl ester N-(N-(4-(Methoxybenzoyl)glycyl)glycine methyl ester (2.70 g, 72%) was prepared in a similar way as N-(4-(methoxybenzoyl)glycine methyl ester, Compound 10, using the N-(glycyl)glycine methyl ester hydrochloride: mp 157 - 159°C; ^1H 400 MHz NMR ($(CD_3)_2SO$) δ 3.65 (3H, s, OCH_3, *Gly'*), 3.83 (3H, s, OCH_3, *Ar*), 3.88 (2H, d, J = 5.8 Hz, CH_2, *Gly'*), 3.91 (2H, d, J = 5.8 Hz, CH_2, *Gly*), 7.03 (2H, d, J = 8.5 Hz, 2H - Ar 2', 6'), 7.89 (2H, d, J = 8.5 Hz, 2H - Ar 3', 5'), 8.35 (1H, t, J = 5.8 Hz, NH, *Gly'*), 8.69 (1H, t, J = 5.9 Hz, NH, *Gly*); mass spectrum (E.I.) *m/z* 281 (28%) (M^+), 165 (100%), 135 (51%). T.L.C. (A), R_f = 0.35. Anal. Found C, 55.50; H, 5.70; N, 9.80. $C_{13}H_{16}N_2O_5$ requires C, 55.70; H, 5.75; N, 10.00.

Compound 30. N-(N-(4-(Methoxybenzoyl)glycyl)glycine ethyl ester N-(N-(4-(Methoxybenzoyl)glycyl)glycine ethyl ester (3.85 g, 30%) was prepared in a similar way as N-(4-(methoxybenzoyl)glycine methyl ester, Compound 10, using the N-(glycyl)glycine ethyl ester hydrochloride: mp 118 - 120°C; ^1H 270 MHz NMR ($(CD_3)_2SO$) δ 1.18 (3H, t, J = 7.9 Hz, $CH_2C\underline{H}_3$), 3.80 (3H, s, OCH_3, *Ar*), 3.83 (2H, d, J = 5.5 Hz, CH_2, *Gly'*), 3.89 (2H, d, J = 5.5 Hz, CH_2, *Gly*), 4.08 (2H, q, J = 7.1 Hz, $C\underline{H}_2CH_3$), 7.00 (2H, d, J = 7.7 Hz, 2H - Ar 2', 6'), 7.86 (2H, d, J = 7.7 Hz, 2H - Ar 3', 5'), 8.30 (1H, brs, NH, *Gly'*), 8.66 (1H, brs, NH, *Gly*); mass spectrum (C.I.) *m/z* 295 (11%) (M + H), 153 (100%), 135 (25%). T.L.C.

(A), $R_f = 0.34$. Anal. Found C, 57.20; H, 6.10; N, 9.10. $C_{14}H_{18}N_2O_5$ requires C, 57.15; H, 6.15; N, 9.50.

Compound 31. N-(N-(4-(Methoxybenzoyl)glycyl)glycine. N-(N-(4-(Methoxybenzoyl)-glycyl)glycine was prepared in a similar way to *Compound 26* to give the product (3.4 g, 84%) as white crystals: mp 231 - 233°C; ^{1}H 270 MHz NMR ($(CD_3)_2SO$) δ 3.76 (2H, d, J = 5.9 Hz, CH_2, *Gly'*), 3.80 (3H, s, OMe), 3.90 (2H, d, J = 5.7 Hz, CH_2, *Gly*), 6.99 (2H, d, J = 8.6 Hz, 2H - Ar 2', 6'), 7.86 (2H, d, J = 8.6 Hz, 2H - Ar 3',5'), 8.20 (1H, t, J = 5.8 Hz, NH, *Gly'*), 8.65 (1H, t, J = 5.9 Hz, NH, *Gly*); T.L.C. (MeOH), $R_f = 0.30$. Anal. Found C, 53.90; H, 5.20; N, 10.40. $C_{12}H_{14}N_2O_5$ requires C, 54.15; H, 5.30; N, 10.50.

Compound 32. N-(N-(4-(Methoxybenzoyl)glycyl)glycine 2,5-dioxopyrrolidin-1-yloxy ester. N-(N-(4-Methoxybenzoyl)glycyl)glycine (1.45 g, 5.45 mmol) was dissolved in DMF (30 mL) and stirred with DCC (1.12 g, 5.45 mmol) for 2h at 0°C. A solution of N-hydroxy-succinimide (0.63 g, 5.45 mmol) was added to this mixture and the whole maintained at 5°C for 16h. A precipitate was removed and the organic phase evaporated to give a residue. Recrystallisation from toluene and DMF gave N-(N-(4-(methoxybenzoyl)glycyl)glycine 2,5-dioxopyrrolidin-1-yloxy ester (1.21 g, 61%) as a white solid: ^{1}H 270 MHz NMR ($(CD_3)_2SO$) δ 2.80 (4H, s, CH_2CH_2), 3.81 (3H, s, OCH_3, Ar), 3.91 (2H, d, J = 6.0 Hz, CH_2, *Gly'*), 4.26 (2H, d, J = 5.9 Hz, CH_2, *Gly*), 7.01 (2H, d, J = 8.8 Hz, 2H - Ar 2', 6'), 7.87 (2H, d, J = 8.8 Hz, 2H - Ar 3', 5'), 8.60 (1H, t, J = 5.8 Hz, NH, *Gly'*), 8.75 (1H, t, J = 5.8 Hz, NH, *Gly*); Anal. Found C, 52.90; H, 4.50; N, 11.65. $C_{16}H_{17}N_3O_7$ requires C, 52.90; H, 4.70; N, 11.55.

Compound 33. N-(N-(1,1-Dimethylethoxycarbonyl)glycyl glycine 2,5-dioxopyrrolidin-1-yloxy ester. N-(N-(1,1-Dimethylethoxycarbonyl)glycyl)glycine (5.95 g, 25.70 mmol) in 1,4-dioxan (190 mL) was treated with DCC (5.30 g, 25.70 mmol) at -10°C for 0.5h. After adding HONSu (2.96 g, 25.70 mmol) the reaction mixture was stored at 5°C for 16h before a precipitate was removed and the solvent evaporated to afford N-(N-(1,1-dimethylethoxy-carbonyl)glycyl glycine 2,5-dioxopyrrolidin-1-yloxy ester as a white solid (8.24 g, 97%): ^{1}H 400 MHz NMR ($(CD_3)_2SO$) δ 1.37 (9H, s, $(CH_3)_3O$), 2.79 (4H, s, CH_2CH_2), 3.58 (2H, d, J = 5.8 Hz, CH_2, *Gly*), 4.24 (2H, d, J = 5.8 Hz, CH_2, *Gly*), 7.02 (1H, t, J = 6.0 Hz, NH), 8.43 (1H, t, J = 5.8 Hz, NH); $C_{13}H_{19}N_3O_7$.

Compound 34. N-(N-(1,1-Dimethylethoxycarbonyl)glycyl)glycine N-(2-(4-methoxyphenyl)ethylamine. A solution of DCC (4.59 g, 22.30 mmol) in DMF (15 mL) was briskly added to N-(N-(1,1-dimethylethoxycarbonyl)glycyl)glycine (5.17 g, 22.30 mmol) in DMF (30 mL) and stirred for 30 min at 0°C. Dropwise 2-(4-(methoxyphenyl)ethylamine (3.06 g, 20.25 mmol) was added to the above and the mixture maintained at 5°C for 16h. Following the removal of a precipitate the filtrate was evaporated under reduced pressure and a residue redissolved in EtOAc. The organic phase was washed with H_2SO_4 (10%), Na_2CO_3 and aqueous NaCl (Sat.). The organic layer was separated, dried and the solvent evaporated to give N-(N-(1,1-dimethylethoxycarbonyl)glycyl glycine N-(2-(4-methoxyphenyl)ethylamine as a white solid (6.75 g, 91%): ^{1}H 270 MHz NMR ($CDCl_3$) δ 1.44 (9H, s, $(CH_3)_3O$), 2.74 (2H, t, J = 7.2 Hz, $CH_2C\underline{H}_2$), 3.44 (2H, q, J = 6.7 Hz, $C\underline{H}_2CH_2$), 3.77 (3H, s, OMe), 3.80 (2H, d, 5.7 Hz, CH_2, *Gly'*), 3.88 (2H, d, J = 5.5 Hz, CH_2, *Gly*), 5.49 (1H, brs, $N\underline{H}CH_2CH_2$), 6.70 (1H, brs, NH, *Gly'*), 6.82 (2H, d, J = 8.6 Hz, 2H - Ar 2', 6'), 7.09 (2H, d, J = 8.6 Hz, 2H - Ar 3', 5'), 7.19 (1H, brs, NH, *Gly*); T.L.C. (A), R_f = 0.64. Anal. Found C, 60.35; H, 7.05; N, 11.15. $C_{18}H_{27}N_3O_5$ requires C, 59.15; H, 7.45; N, 11.50.

Compound 35. N-(1,1-Dimethylethoxycarbonyl)glycine. Glycine (11.81 g, 157 mmol) was dissolved in water (200 mL) containing NaOH (6.29 g, 157 mmol). Di-*t*-butyl dicarbonate (45 g, 204 mmol) in 1,4-dioxan (150 mL) was added to this and the mixture was stirred vigorously for 16h. The reaction mixture was washed with diethyl ether (2 × 100 mL). The aqueous portion was acidified with H_2SO_4 (10%) and extracted with EtOAc (200 mL). The EtOAc was dried and evaporated to yield N-(1,1-dimethylethoxycarbonyl)glycine (25.5 g, 93%) as a white solid: mp 85 - 87°C (lit.[378] mp 88 - 89°C); ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 1.37 (9H, s, $(C\underline{H}_3)_3O$), 3.51 (2H, d, J = 5.9 Hz, $C\underline{H}_2$ *Gly*), 7.81 (1H, brs, NH); mass spectrum (C.I.) *m/z* 176 (8%) (M + H), 120 (100%), 76 (31%), 102 (30%). T.L.C. (A), R_f = 0.40. $C_7H_{13}NO_4$.

Compound 36. N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(4-methoxyphenyl)-ethyl)amide. A mixture of N-(1,1-dimethylethoxycarbonyl)glycine (2.90 g, 16.60 mmol) and DCC (3.13 g, 15.18 mmol) were stirred at 0°C in dry THF (50 mL) for 30 min. A solution of 2-(4-methoxyphenyl)ethylamine (2.09 g, 13.80 mmol) in dry THF was added and the suspension maintained at 5°C for 16h. A precipitate was filtered and the THF was evaporated from the filtrate to give a white solid which was recrystallised from ethanol and water to give N-(1,1-dimethylethoxycarbonyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide

(3.3 g, 78%) as a white solid: mp 116-118°C; ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 1.37 (9H, s, $(CH_3)_3O$), 2.63 (2H, t, J = 7.2 Hz, $HNCH_2CH_2$), 3.29 (2H, q, J = 6.4 Hz, $HNCH_2CH_2$), 3.51 (2H, d, J = 5.9 Hz, CH_2 *Gly*), 3.72 (3H, s, OCH_3), 6.82 (2H, d, J = 8.4 Hz, 2H - Ar 2', 6'), 6.91 (1H, brt, NH), 7.10 (2H, d, J = 8.2 Hz, 2H - Ar 3', 5'), 7.81 (1H, brs, NH); mass spectrum (C.I.) *m/z* 309 (54%) (M + H), 235 (100%), 253 (65%), 134 (86%), 209 (51%). T.L.C. (A), R_f = 0.20. Anal. Found C, 62.50; H, 8.10; N, 9.30. $C_{16}H_{24}N_2O_4$ requires C, 62.30; H, 7.85; N, 9.10.

Compound 37. Glycine N-(2-(4-methoxyphenyl)ethyl)amide. N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide (3.5 g, 11.35 mmol) was dissolved in 1,4-dioxan (80 mL) and acidified with hydrogen chloride. A solid was isolated and partly characterised by ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 2.67 (2H, t, J = 7.3 Hz, $HNCH_2CH_2$), 3.29 (2H, q, J = 6.6 Hz, $HNCH_2CH_2$), 3.49 (2H, brs, CH_2 *Gly*), 3.72 (3H, brs, OCH_3), 6.85 (2H, d, J = 8.4 Hz, 2H - Ar 2', 6'), 7.14 (2H, d, J = 8.1 Hz, 2H - Ar 3', 5'), 8.25 (3H, brs, NH_3), 8.65 (1H, brs, NH). The suspension was filtered and the white solid in water (150 mL), was basified with aq. NaOH (2M) and extracted with $CHCl_3$ (2 × 100mL). Evaporation of the solvent from the dried extract followed by trituration with diethyl ether gave the product glycine N-(2-(4-methoxyphenyl)ethyl)amide (1.79 g, 76%) as a white solid: ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 2.65 (2H, t, J = 7.3 Hz, $HNCH_2CH_2$), 3.27 (2H, q, J = 6.4 Hz, $HNCH_2CH_2$), 3.49 (2H, d, J = 5.5 Hz, CH_2 *Gly*), 3.68 (3H, brs, OCH_3), 6.81 (2H, d, J = 8.4 Hz, 2H - Ar 2', 6'), 7.11 (2H, d, J = 8.4 Hz, 2H - Ar 3', 5'), 8.30 (2H, s, NH_2), 8.71 (1H, t, J = 5.3 Hz, NH); mass spectrum (C.I.) *m/z* 209 (78%) (M + H), 134 (100%), 69 (58%), 183 (26%). T.L.C. (EtOH), R_f = 0.31. Anal. Found C, 63.20; H, 7.35; N, 13.40. $C_{11}H_{16}N_2O_2$ requires C, 63.40; H, 7.75; N, 13.45.

Compound 38. N-(N-(4-Methoxybenzoyl)glycyl)glycine N-(2-(4-methoxyphenyl)-ethyl)amide. Glycine N-(2-(4-methoxyphenyl)ethyl)amide (3.55 g, 14.2 mmol) was added to a solution of N-(4-methoxybenzoyl)glycine pentafluorophenyl ester (5.35 g, 14.2 mmol), N,N-diisopropylethylamine (2.95 g, 22.8 mmol) and HOBt (30 mg) in EtOAc (200 mL). After stirring for 12h a light precipitate was removed by filtration and the solvent was evaporated from the mother liquor. A white solid was collected and recrystallised from MeOH to afford N-(N-(4-methoxybenzoyl)glycyl)glycine N-(2-(4-methoxyphenyl)-ethyl)amide (2.86 g, 50%) as white crystals: mp 199 - 201°C; mass spectrum (FAB [+]) *m/z* 400 (80%) (M + H), 130 (100%), 209 (50%), 192 (39%). (FAB [-]) *m/z* 398 (100%) (M -

H), 552 (50%), 183 (35%), 311 (19%). T.L.C. (MeOH), R_f = 0.21. Anal. Found C, 63.10; H, 6.45; N, 10.70. $C_{21}H_{25}N_3O_5$ requires C, 63.15; H, 6.30; N, 10.50.

Compound 39. N-(1,1-Dimethylethoxycarbonyl)leucine. Leucine (2.00 g, 15.25 mmol) was dissolved in water (25 mL) containing NaOH (0.61 g, 15.25 mmol) and stirred vigorously for 12h with a solution of di-t-butyl dicarbonate (4.98 g, 22.85 mmol) in 1,4-dioxan (15 mL). The reaction was quenched with diethyl ether and the aq. fraction was acidified with H_2SO_4 (10%) and extracted with EtOAc. The solvent was dried and evaporated to afford N-(1,1-dimethylethoxycarbonyl)leucine (3.30 g, 94%) as white crystals: mp 64 - 66°C, (lit. [249] mp 67 - 72°C, softening at 61°C); ^{1}H NMR ($CDCl_3$) 270 MHz δ 1.06 (6H, d, J = 6.3 Hz, $CH(CH_3)_2$), 1.55 (9H, s, $(CH_3)_3O$), 1.78 (3H, m, γ - H, β - H, *Leu*), 4.44 (1H, m, α - H, *Leu*), 5.10 (1H, d, J = 8.5 Hz, NH), 10.11 (1H, s, OH); mass spectrum (C.I.) *m/z* 232 (10%) (M + H), 176 (100%), 132 (42%), 86 (46%). T.L.C. (A), R_f = 0.40. $C_{11}H_{21}NO_4$.

Compound 40. N-(1,1-Dimethylethoxycarbonyl)leucine pentafluorophenyl ester. N-(1,1-Dimethylethoxycarbonyl)leucine (3.45 g, 14.95 mmol) and DCC (3.08 g, 14.95 mmol) were stirred together at -10°C in THF (30 mL) for 1h. Dropwise pentafluorophenol (2.75 g, 14.95 mmol) in THF (40 mL) was added and the suspension was maintained at 5°C for 16h. After removing a precipitate, the solvent was evaporated to afford N-(1,1-dimethylethoxycarbonyl)leucine pentafluorophenyl ester (5.07 g, 85%) as a white solid: mp 48 - 50°C (lit. [332] Mpt 48 - 50°C); ^{1}H NMR ($CDCl_3$) 270 MHz δ 1.06 (6H, d, J = 6.3 Hz, $CH(CH_3)_2$), 1.55 (9H, s, $(CH_3)_3O$), 1.78 (3H, m, γ - H, β - H, *Leu*), 4.44 (1H, m, α - H, *Leu*), 5.10 (1H, d, J = 8.5 Hz, NH); mass spectrum (C.I.) *m/z* 398 (10%) (M + H), 342 (100%), 86 (60%), 225 (40%). T.L.C. (A), R_f = 0.91. $C_{17}H_{20}NO_4F_5$.

Compound 41. N-(N-(1,1-Dimethylethoxycarbonyl)leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide. A solution of Compound 40 (1.80 g, 4.55 mmol) was dissolved in CH_2Cl_2 (30 mL) with N,N-diisopropylethylamine (0.59 g, 4.55 mmol) and HOBt (0.61 g, 4.55 mmol) and stirred as glycine N-(2-(4-(methoxyphenyl)ethyl)amide (Compound 37) (1.10 g, 4.55 mmol) was added. The mixture was stirred for 16h before the organic phase was washed with a minimum amount of water and Na_2HCO_3. The dried extract was evaporated under reduced pressure and the residue separated by column chromatography (CH_2Cl_2 : EtOAc) to give N-(N-(1,1-dimethylethoxycarbonyl)

leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide (0.55 g, 29%) as a white solid: ^{1}H NMR ($CDCl_3$) 400 MHz δ 0.94 (3H, d, J = 6.1 Hz, C*H*$_3$, *Leu*), 0.95 (3H, d, J = 6.4 Hz, C*H*$_3$, *Leu*), 1.45 (9H, s, $(CH_3)_3O$), 1.45 - 1.55 (2H, m, β - H, *Leu*), 1.59 (1H, m, γ - H, *Leu*), 2.75 (1H, dt, J = 14 Hz, J = 7 Hz, CH_2CH_2), 2.76 (1H, dt, J = 14 Hz, J = 7 Hz, CH_2CH_2), 3.45 (2H, q, J = 7 Hz, CH_2CH_2), 3.79 (3H, s, OCH_3), 3.82 (2H, dd, J = 14.5 Hz, J = 5.2Hz, C*H*$_2$, *Gly*), 4.05 (1H, ddd, J = 9.5 Hz, J = 6.7 Hz, J = 5.2 Hz, α - C*H*, *Leu*), 5.01 (1H, d, J = 6.5 Hz, NH, *Leu*), 6.70 (1H, brs, *H*NCH$_2$CH$_2$), 6.84 (2H, d, J = 8.5 Hz, 2H-Ar 3', 5'), 8.14 (1H, m, NH, *Gly*), 7.11 (2H, d, J = 8.5 Hz, 2H-Ar 2', 6'); mass spectrum (C.I.) *m/z* 422 (5%) (M + H), 89 (100%), 322 (10%), 207 (31%). T.L.C. (A), R_f = 0.39. Anal. Found C, 62.60; H, 8.35; N, 9.60. $C_{22}H_{35}N_3O_5$ requires C, 62.70; H, 8.35; N, 10.00.

Compound 42. N-(1,1-Dimethylethoxycarbonyl)phenylalanine 2,5-dioxopyrrolidin-1-yloxy ester. N-(1,1-Dimethylethoxycarbonyl)phenylalanine (4.54 g, 17.12 mmol) in 1,4-dioxan (70 mL) was treated with DCC (3.53 g, 17.12 mmol) at -10°C for 0.5h. After adding HONSu (1.97 g, 17.12 mmol) the reaction mixture was stored at 5°C for 16h before a precipitate was removed and the solvent evaporated to afford a yellow oil. Trituration with diethyl ether gave the product N-(1,1-dimethylethoxycarbonyl)phenylalanine 2,5-dioxopyrrolidin-1-yloxy ester (4.20 g, 68%) as a white solid; mp 152 - 153°C (lit. [250] mp 152-153°C); ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 1.32 (9H, s, $(CH_3)_3$); 2.84 (1H, dd, J = 13.0 Hz, J = 4.00Hz, β - H *Phe*); 2.79 (4H, s, CH_2CH_2), 3.02 (1H, dd, J = 13.0 Hz, J = 4.00Hz, β - H *Phe*); 4.18 (1H, m, α - H *Phe*); 7.09 (1H, d, J 8.4 Hz; N*H*); 7.26 (5H, s, 5H - Ar *Phe*); mass spectrum (C.I.) *m/z* 263 (55%) (M + H), 100 (100%), 307 (10%), 69 (58%), 120 (38%). T.L.C. (EtOAc : light ether, 1 : 1), R_f = 0.82. Anal. Found C, 59.40; H, 6.20; N, 7.85. $C_{18}H_{22}N_2O_6$ requires C, 59.65; H, 6.10; N, 7.75.

Compound 43. N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucine. Aqueous NaOH (30 mL, 2.0 M) and NaHCO3 (8.68 g, 103.32 mmol) were added to a suspension of leucine (15.30 g, 116.70 mmol) in water (200 mL) and stirred for 30 mins before a solution of N-(1,1-dimethylethoxycarbonyl)phenylalanine 2,5-dioxopyrrolidin-1-yloxy ester (42.25 g, 116.70 mmol) (Compound 42) in DMF (250 mL) was added. The mixture was stirred for 1h before water (100 mL) and DMF (100 mL) were added to the reaction mixture. After 16h the semi-solid mass was distributed between EtOAc (200 mL) and H2SO4 (100 mL, 10%) and extracted (three times). The dried extracts were pooled and the organic solvent evaporated to give a pale yellow solid. Recrystallisation from EtOAc and pet. ether

afforded N-(N-(1,1-dimethylethoxycarbonyl)phenylalanyl)leucine (22.95 g, 52%) as a white solid: mp 139 - 141°C (lit. [251] mp 138 - 140°C); ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 0.85 (3H, d, J = 6.4 Hz, C*H*$_3$, *Leu*), 0.90 (3H, d, J = 6.4 Hz, C*H*$_3$, *Leu*), 1.29 (9H, s, (C*H*$_3$)$_3$O), 1.55 (2H, q, J = 7.0 Hz, β H, *Leu*), 1.68 (1H, m, γ H, *Leu*), 2.69 - 2.99 (2H, dd, J = 14 Hz, J = 3.7 Hz, β H, *Phe*), 4.18 (1H, q, J = 6.3 Hz, α H, *Leu*), 4.27 (1H, q, J = 7.5 Hz, α H, *Phe*), 6.86 (1H, d, J = 8.8 Hz, NH, *Phe*), 7.27 (5H, brs, 5H - Ar, *Phe*), 8.10 (1H, d, J = 7.9 Hz, NH, *Leu*); mass spectrum (C.I.) *m/z* 379 (2%) (M + H), 279 (35%), 91 (100%), 305 (20%), 71 (70%). T.L.C. (A), R_f = 0.40. Anal. Found C, 63.20; H, 8.10; N, 7.55. $C_{20}H_{30}N_2O_5$ requires C, 63.50; H, 7.95; N, 7.40; $[\alpha]^{23}_{589}$ = -14.4°.

Compound 44. N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucine pentafluorophenyl ester. N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucine (23.0 g, 61.0 mmol) was stirred with pentafluorophenol (11.2 g, 61.0 mmol) and DCC (12.50 g, 61.0 mmol) in THF (170 mL) for 1h at 0°C before the solution was filtered and the solvent was evaporated from the filtrate. The residue, in CH_2Cl_2 (200 mL), was washed with sat. aq. $NaHCO_3$ (twice) and water. The organic phase was dried and the CH_2Cl_2 was evaporated to give N-(N-(1,1-dimethylethoxycarbonyl)phenylalanyl)leucine pentafluorophenyl ester (28.0 g, 85%) as a yellow solid: ^{1}H NMR ($CDCl_3$) 270 MHz δ 0.96 (6H, d, J = 6 Hz, (C*H*$_3$)$_2$CH *Leu*), 1.41 (9H, s, (C*H*$_3$)$_3$O), 1.71 (3H, m, β - 2H, *Leu*, γ - H, *Leu*), 3.09 (2H, d, J = 6.8 Hz, β - 2H, *Phe*), 4.40 (1H, q, J = 7.5 Hz, α - H, *Leu*), 4.85 (1H, m, α - H, *Phe*), 5.06 (1H, brs, NH, *Leu*), 6.44 (1H, d, J = 7.7 Hz, NH, *Phe*), 7.30 (5H, m, 5H - Ar, *Phenylalanine*); mass spectrum (C.I.) *m/z* 545 (3%) (M + H), 489 (7%), 184 (100%), 120 (45%). T.L.C. (A), R_f = 0.80. Anal. Found C, 57.50; H, 5.55; N, 5.40. $C_{26}H_{29}N_2O_5F_5$ requires C, 57.35; H, 5.35; N, 5.15.

Compound 45. N-(N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide. N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)-leucine pentafluorophenyl ester (30.80 g, 56.60 mmol) was dissolved in dry THF (300 mL) with HOBt (100 mg), DMAP (100 mg) and N,N-diisopropylethylamine (8.05 g, 62.26 mmol). Slowly glycine N-(2-(4-methoxyphenyl)ethyl)amide (11.83 g, 56.60 mmol) in THF (300 mL) was added and the mixture stirred for 16h. The THF was filtered and evaporated to give a residue which, in CH_2Cl_2, was washed with aq. H_2SO_4 (200 mL, 10%) and sat. NaH_2CO_3 (200 mL). Evaporation of the solvent from the dried extract, followed by column chromatography (EtOAc) gave the product .N-(N-(N-(1,1-dimethylethoxycarbonyl)-

phenylalanyl)leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide (22.18 g, 69%) as a pale yellow solid: mp 124-125°C; ^{1}H NMR ($CDCl_3$) 270 MHz δ 0.88 (3H, d, J = 6.0 Hz, CH_3 *Leu*), 0.89 (3H, d, J = 6.2 Hz, C*H*$_3$ *Leu*), 1.38 (9H, s, (C*H*$_3$)$_3$O), 1.45 (1H, m, γ - H *Leu*), 1.49 (1H, m, H - β *Leu*), 1.62 (1H, m, H - β *Leu*), 2.75 (2H, t, J = 7.4 Hz, CH_2C*H*$_2$), 3.43 (2H, qnt, J = 6.05 Hz, C*H*$_2$ *Gly*), 3.03 (2H, q, J = 6.05 Hz, H - β *Phe*), 3.76 (3H, s, OC*H*$_3$), 3.92 (1H, dd, J = 6.1 Hz, J = 5.9 Hz, H - α *Leu*), 4.39 (2H, d, J = 7.1 Hz, C*H*$_2$ *Gly*), 5.22 (1H, d, J = 6.8 Hz, α - H *Phe*), 6.83 (2H, d, J = 8.6 Hz, 2H - Ar 3' - 5'), 7.09 (1H, m, N*H*), 7.14 (8H, m, 2H - Ar 2' - 6', 3H - N*H*'s), 7.24 (2H, d, J = 7.6 Hz, 2H - Ar 2' - 6'); mass spectrum (C.I.) *m/z* 569 (0.75%) (M + H), 134 (100%), 69 (71%), 469 (2%). T.L.C. (A), R_f = 0.38. $C_{31}H_{44}N_4O_6$.

Compound 46. N-(N-(Phenylalanyl)leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide hydrochloride. An excess of hydrogen chloride was bubbled through 1,4-dioxan (400 mL) containing N-(N-(N-(1,1-dimethylethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide (21.26 g, 37.50 mmol). The solvent was evaporated and the residue was triturated with diethyl ether to give N-(N-(phenylalanyl)leucyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide hydrochloride (13.01 g, 69%) as white crystals: mp 224°C; ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 0.87 (3H, d, J = 7.3 Hz, C*H*$_3$, *Leu*), 0.89 (3H, d, J = 7.3 Hz, C*H*$_3$, *Leu*), 1.50 (2H, brt, J = 6.8 Hz, CHC*H*$_2$CH), 1.60 (1H, m, CH_2C*H*$(CH_3)_2$, *Leu*), 2.65 (2H, t, J = 7.4 Hz, CH_2C*H*$_2$), 3.10 (1H, ddd, J = 7.2 Hz, CHC*H*$_2$-Ar, *Phe*), 3.25 (2H, m, C*H*$_2$$CH_2$ and 1H of the ddd @ 3.06), 3.65 (2H, dd, C*H*$_2$, *Gly*), 3.70 (3H, s, OC*H*$_3$), 4.10 (1H, m, α C*H*, *Leu*), 4.30 (1H, q, J = 7.4 Hz, α C*H*, *Phe*), 6.85 (2H, d, J = 8.8 Hz, 2H-Ar 2', 6'), 7.15 (2H, d, J = 8.6 Hz, 2H-Ar 3', 5'), 7.30 (5H, s, 5H-Ar, *Phe*), 7.95 (1H, t, J = 5.5 Hz, HNCH_2CH_2), 8.20 (1H, t, J = 5.6 Hz, HN, *Gly*), 8.40 (3H, m, H_3N, *Phe*), 8.95 (1H, d, J = 7.8 Hz, HN, *Leu*); mass spectrum (FAB [+]) *m/z* 469 (30%) (M + H), 225 (100%), 98 (25%), 209 (11%). (FAB [-]) *m/z* 467 (25%) (M - H), 188 (100%), 151 (60%), 341 (55%), 377 (40%). $C_{26}H_{37}N_4O_4Cl$

Compound 47. N-(1,1-Dimethylethoxycarbonyl)phenylalanine pentafluorophenyl ester. N-(1,1-Dimethylethoxycarbonyl)phenylalanine (2.90 g, 10.95 mmol) and DCC (2.26 g, 10.95 mmol) were stirred together at -10°C in 1,4-dioxan (25 mL) for 1h. Dropwise pentafluorophenol (2.01 g, 10.95 mmol) in 1,4-dioxan (40 mL) was added and the suspension was maintained at 5°C for 16h. After removing the precipitate, the solvent was evaporated to afford N-(1,1-dimethylethoxycarbonyl)phenylalanine pentafluorophenyl ester

(2.85 g, 73%) as a white solid: mp 113 - 115°C (lit. [332] mp 111 - 112°C); ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 1.29 (9H, s, $(CH_3)_3O$), 2.81 (1H, m, β - H *Phe*), 3.01 (1H, m, β - H *Phe*), 4.31 (1H, s, α - H *Phe*), 7.18 (1H, brt, NH *Phe*), 7.25 (5H, m, 5H - Ar *Phe*); mass spectrum (C.I.) *m/z* 376 (100%) (M + H), 164 (62%), 332 (45%), 192 (35%). T.L.C. (CH_2Cl_2 : pentane, [1 : 5]), R_f = 0.30. $[\alpha]^{23}_{569}$ = -26.9°. $C_{20}H_{18}NO_4F_5$.

Compound 49. N-(Phenylalanyl)glycine N-(2-(4-(methoxyphenyl)ethyl)amide hydrochloride. Compound 49 was prepared using a method that was used for the preparation of Compound 39 to give N-(phenylalanyl)glycine N-(2-(4-(methoxyphenyl)ethyl)amide hydrochloride (19.56 g, 89%) as a white solid:

^{1}H NMR ($CDCl_3$) 400 MHz δ 2.75 (1H, dt, J = 14 Hz, J = 7 Hz, CH_2CH_2), 2.76 (1H, dt, J = 14 Hz, J = 7 Hz, CH_2CH_2), 3.03 (2H, q, J = 6.05 Hz, H - β *Phe*), 3.45 (2H, q, J = 7 Hz, CH_2CH_2), 3.79 (3H, s, OCH_3), 3.82 (2H, dd, J = 14.5 Hz, J = 5.2Hz, CH_2, *Gly*), 5.25 (1H, d, J = 6.8 Hz, α - H *Phe*), 6.70 (1H, brs, $HNCH_2CH_2$), 6.84 (2H, d, J = 8.5 Hz, 2H-Ar 3', 5'), 7.11 (2H, d, J = 8.5 Hz, 2H-Ar 2', 6'), 8.14 (1H, m, NH, *Gly*), 8.35 (3H, m, NH_3 *Phe*); T.L.C. ($CHCl_3$: MeOH, [6 : 1]), R_f = 0.78; $C_{20}H_{26}N_3O_3Cl$.

Compound 50. N-(N-(N-(4-Methoxybenzoyl)glycyl)phenylalanyl)glycine N-(2-(4-methoxyphenyl)ethyl)amide. N-(4-Methoxybenzoyl)glycine pentafluorophenyl ester (7.67 g, 20.45 mmol), HOBt (200 mg) and diisopropylethyldiamine (3.12 g, 24.15 mmol) were dissolved in CH_2Cl_2 (300 mL). N-(N-(Phenylalanyl)glycine N-(2-(4-methoxyphenyl)-ethyl)amide (6.60 g, 18.60 mmol) in CH_2Cl_2 (300 mL) was added dropwise to the solution stirred and stirred at 35°C for 24h with DMAP (200 mg). The CH_2Cl_2 was evaporated to give the crude product which was purified using chromatography (EtOAc: CH_2Cl_2 [3 : 1]); $CHCl_3$: MeOH [7 : 1]). The solvent was evaporated and the product N-N-(N-(N-(4-methoxybenzoyl)glycyl)phenylalanyl)leucyl)gycine N-(2-(4-methoxyphenyl)ethyl)amide (4.47 g, 44%) obtained as a white solid: mp 181 - 183°C; ^{1}NMR ($(CD_3)_2SO$) 270 MHz δ 2.58 (2H, t, J = 7.3 Hz, $HNCH_2CH_2$,), 2.83 (1H, dd, J = 13.6 Hz, β - H, *Phe*), 3.03 (1H, dd, J = 13.6 Hz, β - H, *Phe*), 3.17 (2H, m, $HNCH_2CH_2$), 3.63 (2H, dd, J = 5.9 Hz, CH_2, *Gly'*), 3.69 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 3.9 (2H, dd, J = 5.9 Hz, CH_2, *Gly*), 4.46 (1H, m, α - H, *Phe*), 6.80 (2H, d, J = 8.4 Hz, 2H - Ar' 2', 6'), 6.97 (2H, d, J = 9.1 Hz, 2H - Ar 2', 6'), 7.04 (2H, d, J = 8.5 Hz, 2H - Ar' 3', 5'), 7.22 (5H, brs, 5H - Ar, *Phe*), 7.71 (1H, brt, $HNCH_2CH_2$), 7.82 (2H, d, J = 8.5, 2H - Ar 3', 5'), 8.23 (1H, d, J = 7.3 Hz, NH, *Phe*), 8.35

(1H, brt, NH, *Gly'*), 8.61 (1H, brt, NH, *Gly*); mass spectrum (FAB [+]) *m/z* 547 (100%) (M + H), 192 (82%), 149 (55%), 495 (31%). (FAB [-]) *m/z* 545 (100%) (M - H), 493 (42%), 531 (10%). T.L.C. (C), $R_f = 0.25$. $C_{30}H_{34}N_4O_6$.

Compound 51. N-(4-(Phenylmethoxy)benzoyl)glycine methyl ester. 4-(Phenylmethoxy)benzoyl chloride (13.64 g, 55.5 mmol) in CH_2Cl_2 (90 mL) was added dropwise to glycine methyl ester hydrochloride (7.66 g, 61 mmol) and triethylamine (11.78 g, 116.5 mmol) in CH_2Cl_2 (250 mL). The mixture was stirred for 16 h and the suspension filtered. The solvent was evaporated to afford, after recrystallisation from CH_2Cl_2 / hexane, N-(4-(phenylmethoxy)benzoyl)glycine methyl ester (14.75 g, 89%) as a white solid: mp 105 - 107 °C; ^{1}H NMR ($CDCl_3$) 270 MHz δ 3.78 (3H, s, OCH_3), 4.22 (2H, d, J = 5.15 Hz, CH_2, *Gly*), 5.10 (2H, s, $BnCH_2O$), 6.69 (1H, brs, NH), 6.98 (2H, d, J = 8.9 Hz, 2H - Ar 2', 6'), 7.37 - 7.42 (5H, m, *Bn*CH_2O), 7.77 (2H, d, J = 8.9 Hz, 2H - Ar 3', 5'); T.L.C. (heptane : diethyl ether, 1 : 3), $R_f = 0.27$. $C_{17}H_{17}NO_4$.

Compound 52. N-(4-(Phenylmethoxy)benzoyl)glycine. N-(4-(Phenylmethoxy)benzoyl)-glycine methyl ester (14.75 g, 49.2 mmol) was boiled under reflux with methanolic NaOH (1.0 M) (80 mL) for 2 h. The solvent was evaporated. The residue, in water, was acidified (aq. HCl, 5%) and extracted with EtOAc. The extract was washed with brine and the solvent was evaporated from the dried extract to give N-(4-(phenylmethoxy)benzoyl)glycine (6.59 g, 47%) as a white solid: mp 164 - 166°C; ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 3.91 (2H, d, J = 5.9 Hz, CH_2, *Gly*), 5.17 (2H, s, $BnCH_2O$), 7.09 (2H, d, J = 9.1 Hz, 2H - Ar 2', 6'), 7.31 - 7.48 (5H, m, *Bn*CH_2O), 7.85 (2H, d, J = 8.9 Hz, 2H - Ar 3', 5'), 8.71 (1H, t, J = 5.8 Hz, NH), 12.60 (1H, br, OH); mass spectrum (FAB [+]) *m/z* 286 (58%) (M + H), 91 (100%), 211 (58%), 149 (25%). (FAB [-]) *m/z* 284 (100%) (M - H), 97 (80%), 250 (42%). T.L.C. (C), $R_f = 0.30$. $C_{16}H_{15}NO_4$.

Compound 53. N-(4-(Phenylmethoxy)benzoyl)glycine pentafluorophenyl ester. DCC (720 mg, 3.5 mmol) was added to N-(4-(phenylmethoxy)benzoyl)glycine (1.00 g, 3.5 mmol) in dry THF (100 mL) at 0°C. Pentafluorophenol (640 mg, 3.5 mmol) was added dropwise and the mixture was maintained at 0°C for 17h. The suspension was filtered and the solvent was evaporated. The residue, in CH_2Cl_2, was washed with aq. Na_2CO_3 (10%) (75 mL, twice), and with aq. H_2SO_4 (10%). The organic phase was dried and the solvent was evaporated to give a yellow solid, which was recrystallised from CH_2Cl_2 and hexanes to

afford N-(4-(phenylmethoxy)benzoyl)glycine pentafluorophenyl ester (1.5 g, 95%) as white crystals: mp 141 - 143°C, softening at 125°C; ^{1}H NMR ($CDCl_3$) 270 MHz δ 4.59 (2H, d, J = 5.50 Hz, CH_2, Gly), 5.10 (2H, s, BnCH_2O), 6.81 (1H, t, NH), 6.99 (2H, d, J = 8.8 Hz, 2H - Ar 2', 6'), 7.31 - 7.42 (5H, m, *Bn*CH_2O), 7.77 (2H, d, J = 8.8 Hz, 2H - Ar 3', 5'); mass spectrum (FAB [+]) *m/z* 452 (40%) (M + H), 91 (100%), 211 (45%), 225 (20%). (FAB [-]) *m/z* 183 (100%) (M - H). T.L.C. (B), R_f = 0.52. Anal. Found C, 58.70; H, 3.30; N, 3.50. $C_{22}H_{14}NO_4F_5$ requires C, 58.55; H, 3.15; N, 3.10.

Compound 54. N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(4-(phenylmethoxy)-phenyl)ethyl)amide. N-(1,1-Dimethylethoxycarbonyl)glycine (850 mg, 4.85 mmol) was stirred with DCC (1.00 g, 4.85 mmol) and 2-(4-(phenylmethoxy)phenyl)ethylamine (1.00 g, 4.4 mmol) in dry THF (30 mL) for 16 h. The suspension was filtered and the solvent was evaporated to give a residue which, in EtOAc, was washed with aq. H_2SO_4 (10%) and with brine. The solvent was separated from the dried extract give N-(1,1-dimethylethoxy-carbonyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (1.65 g, 98%) as a white solid: mp 107 - 109°C; ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 1.38 (9H, s, $(CH_3)_3O$), 2.62 (2H, t, J = 7.3 Hz, CH_2CH_2), 3.22 (2H, q, CH_2CH_2), 3.48 (2H, d, J = 6.0 Hz, CH_2 *Gly*), 5.06 (2H, s, OCH_2Bn), 6.91 (3H, d, J = 8.4 Hz, 2H - Ar 2', 6', N*H*), 7.11 (2H, d, J = 8.4 Hz, 2H - Ar 3', 5'), 7.31 - 7.42 (5H, m, 5H - OCH$_2$*Bn*), 7.80 (1H, brs, N*H*); mass spectrum (FAB [+]) *m/z* 385 (10%) (M + H), 91 (100%), 210 (45%), 285 (30%). (FAB [-]) *m/z* 383 (20%) (M - H), 309 (100%), 181 (50%), 430 (30%), 537 (40%). T.L.C. (A), R_f = 0.76. Anal. Found C, 69.15; H, 7.10; N, 6.95. $C_{22}H_{28}N_2O_4$ requires C, 68.70; H, 7.35; N, 7.30.

Compound 55. Glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide. N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (2.01 g, 5.23 mmol) was treated with excess HCl in 1,4-dioxan (45 mL) for 2 h. The white solid was collected by filtration and identified as the N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride: mp 196 - 198°C; ^{1}H NMR ($(CD_3)_2SO$) 400 MHz δ 2.68 (2H, t, J = 7.3 Hz, CH_2CH_2), 3.30 (2H, q, J = 6.7 Hz, CH_2CH_2), 3.50 (2H, s, CH_2), 5.07 (2H, s, OCH_2Bn), 6.94 (2H, d, J = 8.3 Hz, 2H - Ar 2', 6'), 7.11 (2H, d, J = 8.3 Hz, 2H - Ar 3', 5'), 7.30 - 7.45 (5H, m, 5H - Ar OCH$_2$*Bn*), 8.31 (3H, s, NH_3), 8.71 (1H, t, J = 5.4 Hz, N*H*); mass spectrum (FAB [+]) *m/z* 285 (100%) (M + H), 91 (50%), 211 (10. (FAB [-]) 188 (100%), 341 (35%), 151 (20%). $C_{17}H_{21}N_2O_2Cl$. The N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride was dissolved in water and EtOAc and the pH was adjusted with aq. NaOH

(2.0 M) to pH 9. The organic phase was dried and the solvent was evaporated to give glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (1.15 g, 77%) as a colourless oil: ^{1}H NMR ($CDCl_3$) 270 MHz δ 2.77 (2H, t, J = 7.0 Hz, CH_2CH_2), 3.30 (2H, s, CH_2 *Gly*), 3.50 (2H, q, J = 6.8 Hz, CH_2CH_2), 5.04 (2H, s, OCH_2Bn), 6.91 (2H, d, J = 8.6 Hz, 2H - Ar 2', 6'), 7.11 (2H, d, J = 8.6 Hz, 2H - Ar 3', 5'), 7.31 - 7.44 (6H, m, 5H - Ar OCH_2*Bn*, NH); T.L.C. ($CHCl_3$: MeOH, [9 : 1]), R_f = 0.40. $C_{17}H_{20}N_2O_2$.

Compound 56. N-(N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide. N,N-Diisopropylethylamine (7.41 g, 57.30 mmol), DMAP (100 mg) and HOBt (100 mg) were stirred for 0.5h with a suspension of glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride (6.73 g, 21.0 mmol) in dry CH_2Cl_2 (350 mL) at 35°C. A solution of N-(N-(1,1-dimethylethoxycarbonyl)-phenylalanyl)leucine pentafluorophenyl ester (10.39 g, 19.10 mmol) in dry CH_2Cl_2 (150 mL) was filtered and added dropwise to the reaction mixture. After 16h the mixture was washed with H_2SO_4 (10%). Evaporation of the solvent from the dried extract followed by column chromatography (Et_2O; EtOAc) afforded N-(N-(N-(1,1-dimethylethoxycarbonyl)-phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide as a yellow oil, which soon crystallised to give a pale yellow solid (8.97 g, 73%): mp 67 - 69°C; ^{1}H NMR ($(CD_3)_2SO$) 400 MHz δ 0.73 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 1), 0.80 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 1), 0.84 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 2), 0.89 (3H, d, J = 6.7 Hz, CH_3 *Leu*, Isomer 2), 1.29 (18H, s, 9H $(CH_3)_3O$, Isomer 1 and 2), 1.42 (2H, m, β - H *Leu*, Isomer 1), 1.49 (2H, m, β - H *Leu*, Isomer 2), 1.60 (1H, m, γ - H *Leu*, Isomer 1), 1.71 (1H, m, γ - H *Leu*, Isomer 2), 2.64 (4H, t, J = 7.5 Hz, $HNCH_2CH_2$, Isomer 1 and 2), 2.81 (1H, m, β - H Phe, Isomer 1), 3.01 (1H, m, β - H *Phe*, Isomer 2), 3.23 (4H, br q, $HNCH_2CH_2$, Isomer 1 and 2), 3.61 (4H, m, CH_2 *Gly*, Isomer 1 and 2), 4.19 (2H, brs, α - H *Leu*, Isomer 1 and 2), 4.31 (2H, s, α - H *Phe*, Isomer 1 and 2), 5.05 (2H, s, CH_2, OCH_2Bn, Isomer 1), 5.06 (2H, s, CH_2, OCH_2Bn, Isomer 2), 6.91 (4H, dd, J = 8.6 Hz, 2H - Ar 2' - 6', Isomer 1 and 2), 6.99 (1H, d, J = 8.3 Hz, NH *Leu*), 7.12 (4H, dd, J = 8.6 Hz, Hz, 2H - Ar 3', 5', Isomer 1 and 2), 7.18 (1H, brt, NH *Phe*, Isomer 1 and 2), 7.25 (10H, m, 5H - Ar *Phe*, Isomer 1 and 2), 7.39 (10H, m, 5H - Ar OCH_2*Bn*, Isomer 1 and 2), 7.81 (1H, t, J = 5.5 Hz, NH *Gly*), 8.06 (1H, t, J = 7.0 Hz, $HNCH_2CH_2$); mass spectrum (FAB [+]) *m/z* 645 (10%) (M + H), 86 (100%), 545 (20%), 285 (35%), 210 (56%). (FAB [-]) *m/z* 643 (8%) (M - H), 569 (100%), 797 (15%), 810 (16%). T.L.C. (C), R_f = 0.62. $[\alpha]^{23}_{589}$. +24.6°. $C_{37}H_{48}N_4O_6$.

Compound 57. N-(N-(N-Phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)-phenyl)ethyl)amide hydrochloride. N-(N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)-leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (3.89 g, 6.05 mmol) was treated with excess hydrogen chloride in CH_2Cl_2 (200 mL) for 3h. The solvent was evaporated to give an oil which was triturated with diethyl ether to give N-(N-(N-phenylalanyl)leucyl)-glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride (3.26 g, 93%) as a white solid mp 108 - 110°C; ^{1}H 400 MHz NMR ($(CD_3)_2SO$) δ 0.69 (3H, d, J = 6.1 Hz, C*H*$_3$ *Leu*, Isomer 1), 0.76 (3H, d, J = 6.1 Hz, C*H*$_3$ *Leu*, Isomer 1), 0.90 (3H, d, J = 6.4 Hz, C*H*$_3$ *Leu*, Isomer 2), 0.93 (3H, d, J = 6.4 Hz, C*H*$_3$ *Leu*, Isomer 2), 1.55 (2H, t, J = 7.3 Hz, β - H *Leu*, Isomer 1), 1.65 (2H, t, J = 7.3 Hz, β - H *Leu*, Isomer 2), 1.67 (1H, m, γ - H *Leu*, Isomer 1), 1.74 (1H, m, γ - H *Leu*, Isomer 2), 2.68 (4H, brs, $HNCH_2CH_2$, Isomer 1 and 2), 3.05 (2H, m, β - H *Phe*, Isomer 1), 3.21 (2H, m, β - H *Phe*, Isomer 2), 3.27 (4H, brs, $HNCH_2CH_2$, Isomer 1 and 2), 3.61 (2H, s, CH_2 *Gly*, Isomer 1), 3.70 (2H, s, CH_2 *Gly*, Isomer 2), 4.15 (2H, brs, α - H *Leu*, Isomer 1 and 2), 4.36 (2H, s, α - H *Phe*, Isomer 1 and 2), 5.10 (4H, brs, CH_2, OCH_2Ph, Isomer 1 and 2), 5.58 (6H, brs, NH_3, Isomer 1 and 2), 6.96 (4H, d, J = 8.2 Hz, 2H - Ar 2', 6'), 7.17 (4H, d, J = 8.2 Hz, 2H - Ar 3', 5'), 7.30 - 7.38 (10H, m, 5H - Ar *Phe*, Isomer 1 and 2), 7.41 - 7.49 (10H, m, 5H - Ar OCH_2*Bn*, Isomer 1 and 2), 7.88 (1H, approx. t, N*H Gly*, Isomer 1), 8.00 (1H, brt, N*H Gly*, Isomer 2), 8.40 (1H, brt, *H*NCH_2CH_2, Isomer 1), 8.66 (1H, brt, *H*NCH_2CH_2, Isomer 1). $C_{32}H_{50}N_4O_4Cl$.

Compound 58. N-(9-Fluorenylmethoxycarbonyl)glutamic acid-γ-(1,1-dimethylethyl)ester-α-pentafluorophenyl ester. In THF (35 mL) a mixture of N-(9-fluorenylmethoxycarbonyl)glutamic acid-γ-(1,1-dimethylethyl)ester (0.92 g, 2.07 mmol) and DCC (0.43 g, 2.07 mmol) was stirred at -10°C for 1h. Pentafluorophenol (0.38 g, 2.07 mmol) in THF (15 mL) was added dropwise and the reaction mixture maintained at 5°C for 2h. The precipitate was removed and the filtrate evaporated to furnish the crude product N-(9-fluorenylmethoxycarbonyl)glutamic acid-γ-(1,1-dimethylethyl)ester-α-pentafluorophenyl ester (1.11 g, 91%) as a white solid; mp 119 - 121°C; ^{1}H 400 MHz NMR ($(CD_3)_2SO$) δ 1.38 (9H, brs, $(CH_3)_3O$), 1.76 (2H, m, β - H *Glu*,), 2.24 (2H, t, J = 6.4 Hz, γ - H *Glu*), 4.02 (1H, m, α - H *Glu*), 4.22 (3H, m, C*H*CH_2, *FMOC*), 7.61 (2H, brt, NH, *Glu*), 7.70 (4H, m, 2H - Ar *FMOC*), 7.89 (4H, d, J = 7.8 Hz, 2H - Ar *FMOC*); ^{19}F 400 MHz NMR ($(CD_3)_2SO$) δ -162.21 (2F, dd, J = 20.8 Hz, J = 17.4 Hz, 2F - meta), -157.63 (1F, t, J = 22.0 Hz, 1F - para), -152.69 (2F, d J = 18.5 Hz, 2F - ortho); T.L.C. ($CHCl_3$), R_f = 0.87. $C_{30}H_{26}NO_6F_5$.

Compound 59. N-(1,1-Dimethylethoxycarbonyl)glycine pentafluorophenyl ester. N-(1,1-Dimethylethoxycarbonyl)glycine (2.00 g, 11.45 mmol) and DCC (2.36 g, 11.45 mmol) were stirred together at -10°C in THF (50 mL) for 1h. Dropwise pentafluorophenol (2.10 g, 11.45 mmol) in THF (40 mL) was added and the suspension was maintained at 5°C for 16h. After removing the precipitate, the solvent was evaporated to afford N-(1,1-dimethylethoxycarbonyl)glycine pentafluorophenyl ester (2.85 g, 73%) as a yellow solid: mp 79 - 80°C (lit. [332] mp 79-80°C); ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 1.40 (9H, s, $(CH_3)_3O$), 4.16 (2H, d, J = 5.8 Hz, Gly), 7.55 (1H, t, J = 5.8 Hz, NH); ^{19}F NMR ($(CD_3)_2SO$) 400 MHz δ -163.95 (2F, dd, J = 23.1 Hz, 19.7 Hz, F - meta), -159.34 (1F, tt, J = 23.2 Hz, F - para), -154.61 (2F, dd, J = 18.5 Hz, F - ortho). $C_{13}H_{12}NO_4F_5$.

Compound 60. N-(N-(N-(N-(1,1-Dimethylethoxycarbonyl)glycyl)phenylalanyl)leucyl)-glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide. N,N-Diisopropylethylamine (0.40 g, 3.10 mmol) was added to a suspension of N-(N-(N-phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride (0.60 g, 1.05 mmol) in dry CH_2Cl_2 (45 mL). The reaction mixture was stirred for 16h with N-(1,1-dimethylethoxycarbonyl)glycine pentafluorophenyl ester (0.35 g, 1.05 mmol), HOBt (30 mg) and DMAP (30 mg). A residue produced when the solvent was evaporated was separated by column chromatography (DCM; EtOAc) and triturated with pentane to give the product N-(N-(N-(N-(1,1-dimethylethoxycarbonyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)-phenyl)ethyl)amide (0.71 g, 96%) as brown crystals: mp 55 - 56°C, softening at 48°C; ^{1}H NMR ($(CD_3)_2SO$) 400 MHz δ 0.67 (3H, d, J = 6.4 Hz, C*H*$_3$, *Leu*, Isomer 1), 0.73 (3H, d, J = 6.1 Hz, C*H*$_3$, *Leu*, Isomer 1), 0.77 (3H, d, J = 6.4 Hz, C*H*$_3$, *Leu*, Isomer 2), (0.81, d, J = 6.4 Hz, C*H*$_3$, *Leu*, Isomer 2), 1.29 (11H, brs, $(CH_3)_3O$, Isomer 1 and 2, CHC*H*$_2$C*H*, *Leu*, Isomer 1), 1.42 (2H, t, J = 7.0 Hz, CHC*H*$_2$CH *Leu*, Isomer 2), 1.54 (1H, m, γ H *Leu*, Isomer 2), 2.58 (2H, q, J = 6.3 Hz, CH_2CH_2, Isomer 1 and 2), 2.95 (2H, dd, J = 13.5 Hz, J = 6.4 Hz, β H *Phe* Isomer 1), 3.04 (2H, dd, J = 13.90 Hz, J = 4.60 Hz, β H *Phe*, Isomer 2), 3.16 (2H, q, J = 6.4 Hz, HNC*H*$_2$CH$_2$, Isomer 1 and 2), 3.35 and 3.65 (2H, m, C*H*$_2$, *Gly* twice, Isomer 1 and 2), 4.09 and 4.21 (1H, m, H - α, *Leu*, Isomer 1 and 2), 4.47 (1H, d, J = 7.3 Hz, H - α, *Phe*, Isomer 1 and 2), 5.03 (2H, s,OC*H*$_2$Bn, Isomer 1 and 2), 6.85 (2H, d, J = 8.6 Hz, 2H - Ar 3' 5', Isomer 1 and 2), 7.05 (2H, d, J = 8.25 Hz, 2H - Ar 2', 6', Isomer 1 and 2), 7.15 (5H, brs, 5H - Ar, *Phe*, Isomer 1 and 2), 7.25 (5H, m, 5H - Ar, OCH$_2$*Bn*, Isomer 1 and 2), 7.60 - 8.25(5H, N*H*, Isomer 1 and 2). T.L.C. (C), R_f = 0.72. $C_{39}H_{51}N_5O_7$.

Compound 61. N-(N-(N-(N-glycylphenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride. An excess of hydrogen chloride was bubbled through a solution of N-(N-(N-((N-(1,1-dimethylethoxycarbonyl)glycyl)-phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (0.61 g, 0.87 mmol) dissolved in CH_2Cl_2 (50 mL) for 1h. The solvent was evaporated under high vacuum to afford the product N-(N-(N-glycylphenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)-phenyl)ethyl)amide hydrochloride. (0.54 g, 98%) as a buff crystalline solid: mp 166 - 168° C; ^{1}H 400 MHz NMR ($(CD_3)_2SO$) δ 0.75 (3H, d, J = 6.4 Hz, CHC*H*$_3$, *Leu*, Isomer 1), 0.80 (3H, d, J = 6.4 Hz, CHC*H*$_3$, *Leu*, Isomer 1), 0.84 (3H, d, J = 6.4 Hz, CHC*H*$_3$, *Leu*, Isomer 2), 0.89 (3H, d, J = 6.4 Hz, CHC*H*$_3$, *Leu*, Isomer 2), 1.41 (1H, m, β - H, *Leu*, Isomer 1), 1.53 (2H, m, β - H, *Leu*, Isomer 2, γ - H Leu, Isomer 1), 1.59 (1H, m, γ - H *Leu*, Isomer 2), 2.64 (4H, brq, J = ?, HNCH_2C*H*$_2$, Isomer 1 and 2), 2.82 (2H, m, β - H, *Phe* Isomer 1 and 2), 3.24 (4H, brq, J = ?, HNC*H*$_2$CH_2, Isomer 1 and 2), 3.58 (4H, m, C*H*$_2$, *Gly*, Isomer 1 and 2), 3.65 (4H, dd, J = 14.4 Hz, J = 5.8 Hz, C*H*$_2$, *Gly*, Isomer 1 and 2), 4.30 (2H, m, α H, *Leu*, Isomer 1 and 2), 4.65 (2H, m, α - H, *Phe*, Isomer 1 and 2), 5.06 (4H, s, OC*H*$_2$Ar, Isomer 1 and 2), 6.92 (4H, d, J = 6.4 Hz, 2H - Ar 2', 6', Isomer 1 and 2), 7.12 (4H, d, J = 7.0 Hz, 2H - Ar 3', 5', Isomer 1 and 2), 7.20 (1H, buried, N*H*), 7.25 (10H, brs, 5H - Ar Phe, Isomer 1 and 2), 7.39 (5H, m, 5H - Ar, OCH$_2$*Ph*, Isomer 1 and 2), 7.93 (1H, brd, NH), 8.11 (3H, brs, N*H*$_3$), 8.51 (1H, brd, N*H*, twice), 8.82 (1H, brd, N*H*); mass spectrum (FAB [+]) *m/z* 602 (49%) (M + H), 149 (100%), 85 (100%), 447 (23%), 285 (20%). (FAB [-]) *m/z* 600 (50%) (M - H), 636 (100%), 188 (95%), 341 (26%). $C_{34}H_{44}N_5O_5Cl$.

Compound 62. N-(N-(N-(N-(N-(9-Fluorenylmethoxycarbonyl)-γ-(1,1-dimethylethyloxy)glutamyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-(methoxyphenyl)phenyl)-ethyl)amide. N,N-Diisopropylethylamine (34.38 g, 0.266 mmol) in dry CH_2Cl_2 (3.0 mL) was added dropwise, over 1h, to a suspension of N-(9-fluorenylmethoxycarbonyl)glutamic acid-γ-(1,1-dimethylethyl)ester-α-pentafluorophenyl ester (78.60 mg, 0.133 mmol) and N-(N-(N-glycylphenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride (84.80 mg, 0.133 mmol) in dry CH_2Cl_2 (15 mL). The solution was stirred for 5h and the residue, following evaporation of the solvent was separated by column chromatography (CH_2Cl_2 : EtOAc [4 : 1]); EtOAc) to furnish N-(N-(N-(N-(N-(9-fluorenyl-methoxycarbonyl)-γ-(1,1-dimethylethyl)glutamyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-(methoxyphenyl)phenyl)ethyl)amide (114 mg, 85%) as pale brown crystals: mp 85 - 87°C; ^{1}H 400 MHz NMR ($(CD_3)_2SO$) δ 0.72 (3H, d, J = 6.3 Hz, CHC*H*$_3$, *Leu*, Isomer 1), 0.78 (3H, d, J = 6.3 Hz, CHC*H*$_3$, *Leu*, Isomer 1), 0.82 (3H, d, J = 6.3 Hz, CHC*H*$_3$, *Leu*, Isomer

2), 0.87 (3H, d, J = 6.8 Hz, CHCH_3, *Leu*, Isomer 2), 1.24 (2H, t, J = 6.1 Hz, β - H *Leu*, Isomer 1), 1.38 (20H, brs, $(CH_3)_3O$, Isomer 1 and 2, γ - H *Leu*, Isomer 1), 1.49 (2H, t, J = 7.0 Hz, β - H *Leu*, Isomer 2), 1.59 (1H, m, γ - H *Leu*, Isomer 2), 1.76 (2H, m, β - H *Glu*, Isomer 1), 1.91 (2H, m, β - H *Glu*, Isomer 2), 2.24 (4H, t, J = 6.4 Hz, γ - H *Glu*, Isomer 1 and 2), 2.64 (4H, t, J = 6.8 Hz, $HNCH_2CH_2$, Isomer 1 and 2), 2.82 (2H, m, β - H *Phe* Isomer 1), 2.96 (2H, m, β - H *Phe*, Isomer 2), 3.24 (4H, q, J = 6.6 Hz, $HNCH_2CH_2$, Isomer 1 and 2), 3.65 (8H, m, CH_2, *Gly*, twice, Isomer 1 and 2), 4.02 (2H, m, α - H *Glu*, Isomer 1 and 2), 4.22 (6H, m, FMOC $CHCH_2$, Isomer 1 and 2), 4.26 (2H, m, α H *Leu*, Isomer 1 and 2), 4.55 (2H, m, α - H *Phe*, Isomer 1 and 2), 5.04 (4H, s,OCH_2Ar, Isomer 1 and 2), 6.92 (4H, d, J = 6.8 Hz, 2H - Ar 2', 6', Isomer 1 and 2), 7.12 (4H, d, J = 6.8 Hz, 2H - Ar 3', 5', Isomer 1 and 2), 7.15 - 7.43 (28H, m, 5H - Ar *Phe*, Isomer 1 and 2, 5H - Ar Bn, Isomer 1 and 2, 4H - *FMOC*, Isomer 1 and 2), 7.61(2H, brt, NH, *Glu*, Isomer 1 and 2), 7.70 (4H, m, 2H - Ar *FMOC*), 7.74 (1H, brt, $HNCH_2CH_2$, Isomer 1), 7.81 (1H, brt, $HNCH_2CH_2$, Isomer 2), 7.89 (4H, d, J = 7.8 Hz, 2H - Ar *FMOC*, Isomer 1 and 2), 7.99 (1H, brt, Glu*Gly*Phe, Isomer 1), 8.05 (1H, d, J = 7.8 Hz, NH *Leu*, Isomer 1), 8.12 (5H, brs, NH *Phe*, Isomer 1 and 2, NH PheLeu*Gly*, Isomer 1 and 2, NH Glu*Gly*Phe, Isomer 2), 8.26 (1H, d, J = 7.8 Hz, NH *Leu*, Isomer 2); mass spectrum (FAB [+]) *m/z* 1009 (7.5%) (M + H), 1031 (1%), 409 (8%). (FAB [-]) *m/z* 1007 (2%) (M - H), 830 (23%), 812 (100%), 785 (70%). Accurate mass. Found 1008.5023, $C_{58}H_{68}N_6O_{10}$ requires 1008.4997. Accurate mass. Found 1009.5100, $C_{58}H_{69}N_6O_{10}$ requires 1009.5075. T.L.C. (C), $R_f = 0.34$. $C_{58}H_{68}N_6O_{10}$.

Compound 63. 2-(2-(2-(2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethoxy)ethyl toluene-4-sulphonate. A solution of hexa(ethyleneglycol) (6 g, 21.5 mmol) in toluene (120 mL) was dried by the azeotropic removal of water before triethylamine (9.69 g, 96.0 mmol), DMAP (250 mg) and tosyl chloride (18.25 g, 9.60 mmol) were added. The mixture was stirred for 36h at 60°C and then allowed to cool. A suspension was removed by filtration and the organic solvent in the filtrate removed by evaporation. A residue was produced which was separated by column chromatography (CH_2Cl_2; CH_2Cl_2 : MeOH [9 : 1]) to afford 2-(2-(2-(2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethoxy)ethyl toluene-4-sulphonate (11%) as an oil: ^1H 270 MHz NMR ($CDCl_3$) δ 2.45 (3H, s, CH_3Ar), 3.67 (20*H*, m, OCH_2CH_2O), 3.75 (4H, m, $TsOCH_2CH_2$, CH_2 - *hydroxy*), 4.15 (2H, t, J = 4.9 Hz, $TsOCH_2$), 7.34 (2H, d, J = 7.9 Hz, 2H - Ar 3', 5'), 7.79 (2H, d, J = 8.3 Hz, 2H - Ar 2', 6'); T.L.C. (B), $R_f = 0.40$. $C_{19}H_{32}O_9$.

Compound 64. 2-(2-(2-(2-Allyloxyethoxy)ethoxy)ethoxy)ethoxy)ethanol. From Dellaria (7). From a flame dried, two necked flask pentaethylene glycol (3.02 g, 12.70 mmol) in dry THF (10 mL) was slowly introduced into a dry mixture of NaH (0.12 g, 5.0 mmol) and dry THF (15 mL) and stirred under nitrogen until all gas evolution had ceased. Fresh allyl bromide (0.61 g, 5.0 mmol) in dry THF (2 mL) was added to the reaction mixture and stirred for 1 h at 75°C. When the reaction had cooled to 40°C the pH was adjusted to pH 2 (glacial acetic acid) and the mother liquor collected after the suspension was filtered through Celite ®. The solvent was evaporated to afford a thick oil which was eluted through a funnel containing silica gel (15 g) using EtOAc, 5% MeOH / EtOAc. The fractions containing the product were combined and the solvent evaporated to afford 2-(2-(2-(2-(2-allyloxyethoxy)ethoxy)ethoxy)ethoxy)ethanol (2.90 g, 82%) as a colourless oil: ^{1}H 270 MHz NMR ($CDCl_3$) δ 3.66 (20H, m, *ethoxy groups*), 4.02 (2H, d, J = 5.7 Hz, CH_2CHCH_2), 5.19 (1H, d, J = 10.4 Hz, CH_2CHCH_2), 5.24 (1H, d, J = 17.4 Hz, CH_2CHCH_2), 5.90 (1H, ddt, J = 17.4 Hz, J = 10.4 Hz, J = 5.8 Hz, CH_2CHCH_2); T.L.C. (C), R_f = 0.43. $C_{13}H_{26}O_6$.

Compound 65. 2-(2-(2-(2-(2-Allyloxyethoxy)ethoxy)ethoxy)ethoxy)ethyl toluene-4-sulphonate. In a method adapted from Dellaria (7) tosyl chloride (0.44 g, 2.35 mmol) was added to a stirred solution of 2-(2-(2-(2-(2-allyloxyethoxy)ethoxy)ethoxy)ethoxy)ethanol (0.54 g, 1.94 mmol), triethylamine (0.31 g, 3.10 mmol) and DMAP (20 mg) in CH_2Cl_2 (15 mL). After 12h the reagents were washed with diethyl ether, aq. HCl (10%), sat. $NaHCO_3$ and brine. The dried extract was evaporated to a thick oil (0.84 g, 74%) which was seperated by column chromatography (EtOAc / pentane [1 : 1]) to give 2-(2-(2-(2-(2-allyl-oxyethoxy)ethoxy)ethoxy)ethoxy)ethyl toluene-4-sulphonate (0.30 g, 36%) as a thick, faintly yellow oil: ^{1}H 270 MHz NMR ($CDCl_3$) δ 2.45(3H, s, CH_3), 3.58 (18H, m, *ethoxy groups*), 4.01 (2H, d, J = 5.7 Hz, CH_2CHCH_2), 4.15 (2H, t, J = 4.8 Hz, $TsOCH_2$), 5.16 (1H, d, J = 10.3 Hz, CH_2CHCH_2), 5.24 (1H, d, J = 17.2 Hz, CH_2CHCH_2), 5.92 (1H, ddt, J = 5.8 Hz, J = 10.3 Hz, J = 17.1 Hz, CH_2CHCH_2), 7.34 (2H, d, J = 8.1 Hz, 2H - Ar 3', 5'), 7.79 (2H, d, J = 8.4 Hz, 2H - Ar 2', 6'); mass spectrum (C.I.) *m/z* 433 (30%) (M + H), 89 (100%), 261 (76%), 279 (20%). T.L.C. (A), R_f = 0.20. $C_{20}H_{32}O_8S$.

Compound 66. Bis(2-(2,5-dioxopyrrolidin-1-yloxycarboxy)ethoxy)ethoxy)ethane. Bis(2-hydroxyethoxy)ethoxy)ethoxy)ethane (5 g, 17.73 mmol), dried by azeotropic removal of toluene was dissolved in toluene / CH_2Cl_2 (3 / 1, 80 mL) and treated with a toluene

solution (20%) of phosgene (91.87 mL, 1.93 M) over 36h. The solution was evaporated to dryness and the remainder of the phosgene removed under vacuum. The residue was redissolved in toluene / CH_2Cl (2 / 1, 100 mL) and treated with N-hydroxysuccinimide (6.12 g, 53.19 mmol) followed by triethylamine (3.77 g, 37.23 mmol). After 3h the solution was filtered and evaporated to dryness. The residue was applied to a chromatography column and a single product was isolated bis(2-(2,5-dioxopyrrolidin-1-yloxycarboxy)ethoxy)-ethoxy)ethane as a yellow oil (2.24 g, 22%): ^1H NMR ($(CD_3)_2SO$) 400 MHz δ 2.81 (8H, s, CH_2CH_2, OSu), 3.35 (4H, s, CH_2CH_2OCO), 3.52 (16H, brs, OCH_2CH_2), 3.70 (4H, CH_2CH_2OCO); T.L.C. ($CHCl_3$: MeOH [6 : 1]), R_f = 0.64. $C_{22}H_{32}N_2O_{15}$.

Compound 67. N-(1,1-Dimethylethoxycarbonyl)sarcosine. Sarcosine (2.00 g, 15.90 mmol) was dissolved in water (25 mL) containing NaOH (1.27 g, 31.85 mmol) and stirred vigorously for 12h with a solution of di-*t*-butyl dicarbonate (5.20 g, 23.90 mmol) in 1,4-dioxan (10 mL). The reaction was quenched with diethyl ether and the aq. fraction was acidified with H_2SO_4 (10%) and extracted with EtOAc. The solvent was dried and evaporated to afford N-(1,1-dimethylethoxycarbonyl)sarcosine (3.00 g, 99%) as buff white crystals: mp 89 - 90°C (lit. [317] mp 89 - 90°C); ^1H NMR ($CDCl_3$) 270 MHz δ 1.45 (9H, s, $(CH_3)_3$), 2.94 (3H, s, CH_3), 3.94 (1H, s, CH), 4.03 (1H, s, CH), 9.50 (1H, s, OH), (there is some restriction of rotation about the N methyl / carbonyl group producing a duplicate singlet peak at 1.48 corresponding to the BOC, and at 2.95 corresponding to the methyl); mass spectrum (C.I.) *m/z* 190 (31%) (M + H), 134 (100%), 90 (72%). T.L.C. (A), R_f = 0.45. $C_8H_{15}NO_4$.

Compound 68. N-(1,1-Dimethylethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester. N-(1,1-Dimethylethoxycarbonyl)sarcosine (10.0 g, 53 mmol) was stirred with 2,4,5-trichlorophenol (10.6 g, 53 mmol) and DCC (10.9 g, 53 mmol) in EtOAc (100mL) at -10°C for 2.5h. The suspension was filtered and the solvent was evaporated. The residue was dissolved in EtOAc and filtered and the solvent was evaporated to give N-(1,1-dimethyl-ethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (19.3 g, 98%) as a white solid: ^1H NMR ($(CD_3)_2SO$) 270 MHz δ 1.40 (9H, s, $(CH_3)_3$), 2.90 (3H, s, CH_3), 3.99 (1H, s, CH), 4.03 (1H, s, CH), 7.32 (1H, s, Ar - 6H), 7.59 (1H, s, Ar - 3H); T.L.C. (A), R_f = 0.88. $C_{14}H_{16}NO_4Cl$.

Compound 69. N-(1,1-Dimethylethoxycarbonyl)sarcosine N-(2-aminoethyl)amide. N-(1,1-Dimethylethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (12.7 g, 34.5 mmol) in CH_2Cl_2 (50 mL) was added during 30 min to ethane-1,2-diamine (20.7 g, 345 mmol) in CH_2Cl_2 (150 mL) and the solution was stirred for 2 h. The solution was washed with water (2 × 75 mL) and with aq. $NaCO_3$ (10%; 50 mL) and dried. The solvent was evaporated to give N-(1,1-dimethylethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (6.9 g, 86%) as a yellow foam: ^1H NMR ($(CD_3)_2SO$) 270 MHz δ 1.40 (9H, s, $(CH_3)_3O$), 2.65 (2H, t, J = 6.4 Hz, CH_2CH_2), 2.80 (3H, s, NCH_3), 3.10 (2H, q, J = 6.1 Hz, CH_2CH_2), 3.70 (1H, s, CH, Sar), 3.75 (1H, s, CH, Sar), 4.00 (2H, brs, NH_2), 7.90 (1H, s, NH); (FAB [+]) *m/z* 232 (100%) (M + H), 172 (70%), 438 (40%), 215 (33%). T.L.C. (MeOH), R_f = 0.20. $C_{10}H_{21}N_3O_3$.

Compound 70. Bis(2-(2-(N-(2-(N-(1,1-Dimethylethoxycarbonyl)sarcosyl)aminoethyl)-aminocarboxy)ethoxy)ethoxy)ethane. Bis(2-hydroxyethoxy)ethoxy)ethane (5.0 g, 21 mmol), dried by azeotropic removal of water with toluene (120 mL), was dissolved in toluene (120 mL) / CH_2Cl_2 (40 mL) and treated with a solution of phosgene in toluene (1.93 M) (109 mL, 210 mmol) for 4h. An aliquot (30 mL) of this solution was evaporated to yield the known compound (279, 283) bis(2-(2-(chlorocarboxy)ethoxy)ethoxy)ethane (900 mg, 2.5 mmol), which was re-dissolved in DCM (50mL) and added to N-(1,1-dimethylethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (1.73 g, 7.5 mmol) in DCM (100 mL). Triethylamine (1.26 g, 12.5 mmol) and DMAP (20 mg) were added to the solution and the mixture stirred for 6h, washed with H_2O, aq. H_2SO_4 (10%) and H_2O. Evaporation of the dried extract afforded the product bis(2-(2-(N-(2-(N-(1,1-dimethylethoxycarbonyl)-sarcosyl)aminoethyl)aminocarboxy)ethoxy)ethoxy)ethane(1.1 g, 59%) as a yellow oil: ^1H NMR ($CDCl_3$) 270 MHz δ 1.38 (9H, s, $(CH_3)_3O$), 2.86 (6H, s, NCH_3, *Sarc*), 3.23 (4H, brs, CH_2CH_2, CH_2CH_2), 3.31 (4H, brs, CH_2CH_2, CH_2CH_2), 3.58 (16H, brs, CH_2 - 2, 3, 4, 5, 6, 7, 8, 9), 3.78 (4H, s, CH_2 - 1, 10), 4.13 (4H, s, CH_2, *Sarc*), 5.53 (1H, brs, HN'), 5.83 (1H, brs, HN""), 6.78 (1H, brs, HN"), 6.93 (1H, brs, HN"'); $C_{32}H_{60}N_6O_{14}$.

Compound 71. Bis(2-(2-(N-(2-sarcosyl)aminoethyl)aminocarboxy)ethoxy)ethoxy)-ethane hydrochloride. An excess of hydrogen chloride was bubbled through CH_2Cl_2 (30 mL) containing bis(2-(2-(N-(2-(N-(1,1-dimethylethoxycarbonyl)sarcosyl)aminoethyl)-aminocarboxy)ethoxy)ethoxy)ethane (300 mg, 0.40 mmol) (Compound 70). The solvent was evaporated to afford Bis(2-(2-(N-(2-sarcosyl)aminoethyl)aminocarboxy)ethoxy)-

ethoxy)ethane hydrochloride (136 mg, 55%) as a yellow oil: ^{1}H NMR ($(CD_3)_2SO$) 400 MHz δ 2.53 (6H, t, J = 5.3 Hz, NCH_3), 3.07 (4H, q, J = 5.8 Hz, CH_2CH_2 - 4,17), 3.14 (4H, q, J = 5.8 Hz, CH_2 - 3, 18), 3.55 (16H, m, CH_2 - 7 - 14), 3.63 (4H, t, J = 5.7 Hz, CH_2 - 1, 20), 4.04 (4H, t, J = 4.6 Hz, CH_2 - 6, 15), 7.28 (2H, m, NH, coupled to 3.07), 8.62 (2H, m, NH, coupled to 3.14), 9.00 (4H, m, NH_2); mass spectrum (FAB [+]) *m/z* 553 (40%) (M + H), 495 (100%), 414 (83%), 144 (70%), 396 (60%). (FAB [-]) *m/z* 188 (100%), 341 (29%), 448 (12%), 529 (10%). $C_{22}H_{44}N_6O_{10}Cl_2$.

Compound 72. A POLYMER. 4-(Oxiranylmethoxy)-N-(2-(4-oxiranylmethoxy)phenyl)-ethyl)benzamide (140 mg, 0.81 mmol) and decane-1,10-diamine (300 mg, 0.81 mmol) were suspended in EtOH (20 mL) and boiled under reflux for 36h. The solution was allowed to cool and the filtrate was evaporated to give an oil (430 mg, 77%): mass spectrum (FAB [+]) *m/z* 542 (100%) (M + H), 1083 (8%), 912 (10%), 173 (92%), 89 (95%). $C_{31}H_{47}N_3O_5$.

Compound 73. N-(2-Methoxyethyl)formamide. Ethyl formate (29.63 g, (32.31 mL), 400 mmol) was added to 2-methoxyethylamine (15 g, 200 mmol) in dry absolute ethanol (450 mL) and stirred under nitrogen for 6h. Distillation (140°C, 20 torr) of the reaction mixture afforded N-(2-methoxyethyl)formamide (16.20 g, 79%) as a colourless liquid: ^{1}H NMR ($CDCl_3$) 270 MHz δ 3.25 (3H, brs, OCH_3), 3.37 (4H, brs, CH_2CH_2), 6.67 (1H, m, NH), 8.06 (1H, s, HNCOH); T.L.C. (A), R_f = 0.58. $C_4H_9NO_2$.

Compound 74. N-Methyl-2-methoxyethylamine. A solution of N-(2-methoxy-ethyl)formamide (7.00 g, 68 mmol) in dry THF (30 mL) was carefully added to a suspension of $LiAlH_4$ (6.46 g, 170 mmol) boiling under reflux in dry THF (50 mL). After 8h the reaction was quenched with water (30 mL) and extracted with diethyl ether (100 mL, × 3). The extract, after drying with K_2CO_3, was distilled (95 - 97°C) to afford N-methyl-2-methoxyethylamine (1.00 g, 15%) as a colourless liquid: (lit. [325] Bpt 98-99°C); ^{1}H NMR ($CDCl_3$) 270 MHz δ 1.60 (1H, brs, NH), 2.45 (3H, s, CH_3), 2.74 (2H, t, J = 5.1 Hz, CH_2CH_2), 3.36 (3H, brs, CH_3O), 3.49 (2H, t, J = 5.1 Hz, CH_2CH_2); T.L.C. (A), R_f = 0.45. $C_4H_{11}NO$.

Compound 75. 4-(2-Hydroxy-3-(N-(2-methoxyethyl)methylamino)propoxy) N-(2-(4-(2-hydroxy-3-(N-(2-methoxyethyl)methylamino)propoxy)phenyl)ethyl)benzamide. 4-(Oxiranylmethoxy) N-(2-(4-oxiranylmethoxy)phenyl)ethyl)benzamide (100 mg, 0.27 mmol) was dissolved in absolute ethanol (15 mL) and N-methyl-2-methoxyethylamine (300 mg, 2.96 mmol) added and the mixture was boiled under reflux for 2h. Evaporation of the solvent gave the product 4-(2-hydroxy-3-(N-(2-methoxyethyl)methylamino)propoxy) N-(2-(4-(2-hydroxy-3-(N-(2-methoxyethyl)methylamino)propoxy)phenyl)ethyl)benzamide (75 mg, 51%) as a gum: ^{1}H NMR ($(CD_3)_2SO$) 500 MHz δ 2.23 (3H, s, N(CH_3)), 2.24 (3H, s, N(CH_3)), 2.34 - 2.58 (8H, m, CH_2), 2.75 (2H, t, J = 7.5 Hz, $NHCH_2CH_2$), 3.19 (3H, s, O(CH_3)), 3.20 (3H, s, O(CH_3)), 3.35 - 3.39 (10H, m, CH_2), 3.78 - 3.94 (2H, m, OH*H*), 4.77 (1H, brs, OH), 4.84 (1H, brs, OH), 6.85 (2H, d, J = 8.5 Hz, 2H - Ar' 2', 6'), 6.97 (2H, d, J = 8.5 Hz, 2H - Ar 2', 6'), 7.13 (2H, d, J = 8.5 Hz, 2H - Ar' 2', 6'), 7.78 (2H, d, J = 8.5 Hz, 2H - Ar 3', 5'), 8.38 (1H, m; NH); mass spectrum (C.I.) *m/z* 548 (62%) (M + H), 102 (100%), 445 (25%), 146 (30%), 590 (6%). T.L.C. ($CHCl_3$: MeOH [5 : 1]), R_f = 0.45. $C_{29}H_{45}N_3O_7$.

Compound 76. 1,10-Bis(formamido)decane. Ethyl formate (4.30 g, 58.00 mmol) and anhydrous potassium carbonate (0.81 g, 5.80 mmol) were added to decane-1,10-diamine (0.50 g, 2.90 mmol) in dry absolute ethanol (100 mL) and stirred for 8h. A precipitate was removed and the solvent was evaporated from the filtrate to give a residue. The residue was taken up in CH_2Cl_2 (60 mL) and filtered, the solvent was evaporated to afford 1,10-bis-(formamido)decane (0.42 g, 64%) as a an oil; ^{1}H 400 MHz NMR ($CDCl_3$) δ 1.20 (12H, s, H - 1, 3, 4, 5, 6, 7, 8), 1.52 (4H, m, H - 2, 9), 3.30 (4H, q, J = 6.7 Hz, H - 1, 10), 5.80 (2H, brs, HCON*H* 1, 10), 8.16 (2H, s, *H*CONH, 1, 10); mass spectrum (C.I.) *m/z* 229 (100%) (M + H), 69 (19%), 201 (15%). Anal. Found C, 63.20; H, 10.80; N, 12.20. $C_{12}H_{24}N_2O_2$ requires C, 63.10; H, 10.60; N, 12.25.

Compound 77. N,N'-Dimethyldecane-1,10-diamine. A mixture of 1,10-bis(formamide)decane (0.5 g, 2.21 mmol) in dry THF (30 mL) was carefully added to a suspension of $LiAlH_4$ (0.48 g, 12.75 mmol) boiling under reflux in dry THF (50 mL). After 8h the reaction was quenched with water (5 mL) and a solid removed by filtration. The filtrate was evaporated to afford N,N'-dimethyldecane-1,10-diamine (0.37 g, 86%) as a colourless oil: 2.50 (2H, m, HCON*H* 1, 10), ^{1}H 400 MHz NMR ($CDCl_3$) δ 1.20 (12H, s, H - 1, 3, 4, 5, 6, 7, 8), 1.52 (4H, m, H - 2, 9), 2.53 (6H, s, NCH_3), 2.70 (4H, q, J = 6.7 Hz, H -

1, 10), 5.80 (2H, brs, N*H* 1, 10); mass spectrum (FAB [+]) *m/z* 201 (100%), 243 (10%), 157 (9%). (FAB [-]) *m/z* 199 (20%) (M + H), 201 (100%), 213 (40%). $C_{12}H_{28}N_2$.

Compound 78. A POLYMER. 4-(Oxiranylmethoxy)-N-(2-(4-oxiranylmethoxy)phenyl)-ethyl)benzamide (0.46 g, 1.25 mmol) and N,N'-dimethyldecane-1,10-diamine (0.25 g, 1.25 mmol) were suspended in EtOH (20 mL) and boiled under reflux for 16h. The solution was allowed to cool and a solid was collected by filteration (0.24 g, 34%). The organic solvent in the filtrate was evaporated to afford an oil (0.37 g, 52%): $C_{33}H_{51}N_3O_5$. The products were not characterised any further.

Compound 79. N-(N-(N-(4-(Oxiranylmethoxy)benzoyl)phenylalanyl)leucyl)glycine N-(2-(4-(oxiranylmethoxy)phenyl)ethyl)amide.

N-(N-(N-(4-Hydroxybenzoyl)phenylalanyl)leucyl)glycine N-(2-(4-hydroxyphenyl)ethyl)-amide (1.05 g, 1.82 mmol) was suspended in water (10 mL) containing NaOH (0.73 g, 18.25 mmol). Epichlorohydrin (8.44 g, 91.23 mmol) in MeOH (35 mL) was added, followed by benzylammonium hydroxide (21 drops, 40% solution in water) and the solution was stirred at 40°C for 48h. The solvent and epichlorohydrin were evaporated under high vacuum and the residue was partitioned between water and EtOAc. The EtOAc was dried and evaporated. Column chromatography ($CHCl_3$; $CHCl_3$: MeOH [100 : 1]; $CHCl_3$: MeOH [100 : 5]) afforded N-(N-(N-(4-(oxiranylmethoxy)benzoyl)phenylalanyl)-leucyl)glycine N-(2-(4-(oxiranylmethoxy)phenyl)ethyl)amide as a white solid (1.11 g, 89%): mp 73 - 75°C; ^{1}H NMR ($(CD_3)_2SO$) 400 MHz δ 0.76 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 1), 0.82 (3H, d, J = 6.7 Hz, CH_3 *Leu*, Isomer 1), 0.84 (3H, d, J = 6.4 Hz, CH_3 *Leu*, Isomer 2), 0.88 (3H, d, J = 6.7 Hz, CH_3 *Leu*, Isomer 2), 1.32 (1H, m, γ - H *Leu*, Isomer 1), 1.44 (2H, t, J = 7.2 Hz, β - H *Leu*, Isomer 1), 1.52 (2H, t, J = 7.2 Hz, β - H *Leu*, Isomer 2), 1.60 (1H, m, γ - H *Leu*, Isomer 2), 2.52 (2H, buried, $HNCH_2CH_2$, Isomer 1), 2.63 (2H, t, J = 7.5 Hz, $HNCH_2CH_2$, Isomer 2), 2.69 (2H, m, CH - 3, *Oxiranyl*, Isomer 1 and 2), 2.84 (2H, m, CH - 3, *Oxiranyl*, Isomer 1 and 2), 2.99 (4H, m, β - H *Phe*, Isomer 1 and 2), 3.12 (2H, m, $HNCH_2CH_2$, Isomer 1), 3.25 (2H, m, $HNCH_2CH_2$, Isomer 2), 3.30 (2H, m, CH - 2, *Oxiranyl*, Isomer 1 and 2), 3.61 (2H, m, CH_2 *Gly*, Isomer 1 and 2), 3.75 (2H, m, CH, CH_2 *Oxiranyl*, Isomer 1 and 2), 3.84 (2H, m, CH, CH_2 *Oxiranyl*, Isomer 1 and 2), 4.19 (1H, q, J = 7.3 Hz, α - H *Leu*, Isomer 1), 4.25 (1H, q, J = 7.3 Hz, α - H *Leu*, Isomer 2), 4.28 (2H, m, CH, CH_2 *Oxiranyl*, Isomer 1 and 2), 3.30 (2H, m, CH, CH_2 *Oxiranyl*, Isomer 1 and 2), 4.66 (1H, q, J = 7.6 Hz, α - H *Phe*, Isomer 1), 4.73 (1H, m, α - H *Phe*, Isomer 2), 6.81 (4H, dd,

J = 8.5 Hz, J = 8.5 Hz, 2H - Ar' 2', 6', Isomer 1 and 2), 7.00 (4H, dd, J = 8.9 Hz, J = 8.9 Hz, 2H - Ar 2', 6'), 7.09 (4H, dd, J = 8.5 Hz, J = 8.5 Hz, 2H - Ar' 3', 5'), 7.34 (10H, brs, 5H - Ar *Phe*, Isomer 1 and 2), 7.60 (2H, brs, $\underline{H}NCH_2CH_2$, Isomer 1 and 2), 7.79 (4H, dd nature, J = 8.9 Hz, J = 8.9 Hz, 2H - Ar 3', 5'), 8.13 (1H, t, J = 5.6 Hz, NH *Gly*, Isomer 1), 8.19 (1H, d, J = 7.3 Hz, NH *Leu*, Isomer 2), 8.27 (1H, t, J = 5.6 Hz, NH *Gly*, Isomer 2), 8.44 (1H, d, J = 7.6 Hz, NH *Leu*, Isomer 1), 8.60 (2H, d, J = 7.3 Hz, NH *Phe*, Isomer 1 and 2); mass spectrum (FAB [+]) *m/z* 687 (16%) (M + H), 177 (100%), 709 (2%), 723 (11%). (FAB [-]) *m/z* 684 (100%) (M - 2H), 721 (52%), 756 (15%), 628 (30%). Accurate mass. Found 687.3383, $C_{38}H_{47}N_4O_8$ requires 687.3394. T.L.C. ($CHCl_3$: MeOH [1 : 1])), R_f = 0.60. $C_{38}H_{46}N_4O_8$.

Compound 80. 4-(Oxiranylmethoxy)-N-(2-(4-oxiranylmethoxy)phenyl)ethyl)-benzamide. 4-Hydroxy-N-(2-(4-hydroxyphenyl)ethyl benzamide (300 mg, 1.17 mmol) was dissolved in water (0.30 mL) containing NaOH (100 mg, 2.34 mmol) and added to an excess of epichlorohydrin (10.82 g, 117 mmol). The reaction mixture was warmed to 60°C and benzyltrimethylammonium hydroxide (200 mg, 1.17 mmol) was added to the solution. The mixture was for 16h and then, in CH_2Cl_2, was washed with water and the solvent was evaporated from the dried extract to give a residue. The product, 4-(oxiranylmethoxy)-N-(2-(4-oxiranylmethoxy)phenyl)ethyl)benzamide (290 mg, 67%) was afforded after column chromatography (EtOAc / CH_2Cl_2 (10:1)) as a buff solid: mp 153 - 154°C; ^{1}H NMR ($(CD_3)_2SO$) 270 MHz δ 2.69 (1H, dd, J = 5.2 Hz, J = 2.7 Hz, H - 3 *Oxiranyl*), 2.71 (1H, dd, J = 5.2 Hz, J = 2.7 Hz, H - 3 *Oxiranyl*), 2.76 (2H, t, J = 7.5 Hz, $CH_2C\underline{H_2}$), 2.83 (1H, t, J = 4.7 Hz, H - 3 *Oxiranyl*), 2.85 (1H, t, J = 4.7 Hz, H - 3 *Oxiranyl*), 3.31 (1H, m, H - 2 *Oxiranyl*), 3.34 (1H, m, H - 2 *Oxiranyl*), 3.42 (2H, q, J = 6.8 Hz, $C\underline{H_2}CH_2$), 3.78 (1H, dd, J = 11.3 Hz, J = 6.7 Hz, H - *CH_2OAr Oxiranyl*), 3.88 (1H, dd, J = 11.3 Hz, J = 6.7 Hz, H - *CH_2OAr Oxiranyl*), 4.27 (1H, dd, J = 11.3 Hz, J = 2.4 Hz, H - *CH_2OAr Oxiranyl*), 4.39 (1H, dd, J = 11.3 Hz, J = 2.4 Hz, H - *CH_2OAr Oxiranyl*),6.88 (2H, d, J = 8.5 Hz, 2H-Ar' 2' 6'), 7.01 (2H, d, J = 8.9 Hz, 2H-Ar 2', 6'), 7.15 (2H, d, J = 8.5 Hz, 2H-Ar' 3', 5'), 7.80 (2H, d, J = 8.9 Hz, 2H-Ar 3', 5'), 8.41 (1H, t, J = 5.5 Hz, NH); mass spectrum (E.I.) *m/z* 370 (30%) (M^+), 89 (100%), 176 (60%), 57 (48%). T.L.C. (A), R_f = 0.55. Anal. Found C, 68.50; H, 6.30; N, 3.85. $C_{21}H_{23}NO_5$ requires C, 68.30; H, 6.30; N, 3.80.

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